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(54) **EXPRESSION OF LIPOPROTEINS**
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(56) References cited:
WO-A-91/09952 **WO-A-92/00055**
WO-A-92/14488 **WO-A-93/08306**
WO-A-94/14318 **US-A- 4 624 926**

- **STOVER, C.K. ET AL.: "Protective Immunity Elicited by Recombinant Bacille Calmette-Guerin (BCG) Expressing Outer Surface Protein A (OspA) Lipoprotein: A Candidate Lyme Disease Vaccine" J. EXP. MED., vol. 178, July 1993 (1993-07), pages 197-209, XP002002895**
- **ERDILE, L.F. ET AL.: "Role of Attached Lipid in Immunogenicity of Borrelia burgdorferi OspA" INFECT. IMMUN., vol. 61, no. 1, January 1993 (1993-01), pages 81-90, XP002007535**
- **INFECTION AND IMMUNITY, May 1994, Vol. 62, No. 5, PROBERT W.S. et al., "Protection of C3H/HeN Mice from Challenge with Borrelia burgdorferi Through Active Immunization with OspA, OspB, or OspC, but not with OspD or the 83-Kilodalton Antigen", pages 1920-1926.**
- **INFECTION AND IMMUNITY, November 1994, Vol. 62, No. 11, FERRERO R.L. et al., "Recombinant Antigens Prepared from the Urease Subunits of Helicobacter Spp.: Evidence of Protection in a Mouse Model of Gastric Infection", pages 4981-4989.**

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DescriptionREFERENCE TO RELATED APPLICATIONSFIELD OF THE INVENTION

[0001] The present invention is concerned with genetic engineering to effect expression of OspC lipoproteins of a *Borrelia* species from vectors containing nucleic acid molecules encoding the lipoproteins. More particularly, the present invention relates to expression of a recombinant OspC lipoprotein of a *Borrelia* species wherein the lipidation thereof is from expression of a first nucleic acid sequence encoding an OspA leader sequence of a *Borrelia* species and the protein thereof is from expression of a second nucleic acid sequence, the first and second nucleic acid sequences, which do not naturally occur together, being contiguous. The invention also relates to recombinant lipidated OspC proteins of a *Borrelia* species expressed using the nucleic acid sequence encoding the OspA leader sequence of a *Borrelia* species, methods of making and using the same compositions thereof and methods of using the compositions. The invention additionally relates to nucleic acid sequences encoding the recombinant OspC lipoproteins of a *Borrelia* species, vectors containing and/or expressing the sequences, methods for expressing the lipoproteins and methods for making the nucleic acid sequences and vectors; compositions employing the lipoproteins, including immunogenic or vaccine compositions, such compositions preferably having improved immunogenicity; and methods of using such compositions to elicit an immunological or protective response.

BACKGROUND OF THE INVENTION

[0002] Lyme borreliosis is the most prevalent tick-borne disease in the United States as well as one of the most important tick-borne infectious diseases worldwide. The spirochete *Borrelia burgdorferi* is the causative agent for Lyme disease. Infection with *B. burgdorferi* produces local and systemic manifestations. Local symptoms that appear early after infection are a skin lesion at the site of the tick bite, termed erythema migrans. Weeks to months after infection, systemic manifestations that include rheumatic, cardiac and neurological symptoms appear. The early local phase of *B. burgdorferi* infection is easily treatable with antibiotics. However, the later systemic phases have proved to be more refractory to antibiotics.

[0003] Substantial effort has been directed toward the development of a vaccine for Lyme disease. Two distinct approaches have been used for vaccine development. One approach is to use a vaccine composed of whole inactivated spirochetes, as described by Johnson in U.S. Pat. No. 4, 721, 617. A whole inactivated vaccine has been shown to protect hamsters from challenge and has been licensed for use in dogs.

[0004] Due to the concerns about cross-reactive antigens within a whole cell preparation, human vaccine research has focused on the identification and development of non-cross-reactive protective antigens expressed by *B. burgdorferi*. Several candidate antigens have been identified to date. Much of this effort has focused on the most abundant outer surface protein of *B. burgdorferi*, namely outer surface protein A (OspA), as described in published PCT patent application WO 92/14488, assigned to the assignee hereof. Several versions of this protein have been shown to induce protective immunity in mouse, hamster and dog challenge studies. Clinical trials in humans have shown the formulations of OspA to be safe and immunogenic in humans [Keller et al., JAMA (1994) 271:1764-1768]. Indeed, one formulation containing recombinant lipidated OspA as described in the aforementioned WO 92/14488, is now undergoing Phase III safety/efficacy trials in humans.

[0005] While OspA is expressed in the vast majority of clinical isolates of *B. burgdorferi* from North America, a different picture has emerged from examination of the clinical *Borrelia* isolates in Europe. In Europe, Lyme disease is caused by three genospecies of *Borrelia*, namely *B. burgdorferi*, *B. garinii* and *B. afzelii*. In approximately half of the European isolates, OspA is not the most abundant outer surface protein. A second outer surface protein C (OspC) is the major surface antigen found on these spirochetes. In fact, a number of European clinical isolates that do not express OspA have been identified. Immunization of gerbils and mice with purified recombinant OspC produces protective immunity to *B. burgdorferi* strains expressing the homologous OspC protein [V. Preac-Mursic et al., INFECTION (1992) 20:342-349; W. S. Probert et al., INFECTION AND IMMUNITY (1994) 62:1920-1926]. The OspC protein is currently being considered as a possible component of a second generation Lyme vaccine formulation.

[0006] Recombinant proteins are promising vaccine or immunogenic composition candidates, because they can be produced at high yield and purity and manipulated to maximize desirable activities and minimize undesirable ones. However, because they can be poorly immunogenic, methods to enhance the immune response to recombinant proteins are important in the development of vaccines or immunogenic compositions.

A very promising immune stimulator is the lipid moiety N-palmitoyl-S-(2RS)-2,3-bis-(palmitoyloxy)propyl-cysteine, abbreviated Pam₃Cys. This moiety is found at the amino terminus of the bacterial lipoproteins which are synthesized with a signal sequence that specifies lipid attachment and cleavage by signal peptidase II. Synthetic peptides that by them-

selves are not immunogenic induce a strong antibody response when covalently coupled to Pam₃Cys [Bessler et al., Research Immunology (1992) 143:548-552].

[0007] In addition to an antibody response, one often needs to induce a cellular immune response, particularly cytotoxic T lymphocytes (CTLs). Pam₃Cys-coupled synthetic peptides are extremely potent inducers of CTLs, but no one has yet reported CTL induction by large recombinant lipoproteins

[0008] The nucleic acid sequence and encoded amino acid sequence for OspA are known for several *B. burgdorferi* clinical isolates and is described, for example, in published PCT application WO 90/04411 (Symbicom AB) for B31 strain of *B. burgdorferi* and in Johnson et al., Infect. Immun. 60:1845-1853 for a comparison of the *ospA* operons of three *B. burgdorferi* isolates of different geographic origins, namely B31, ACA1 and Ip90.

[0009] As described in WO 90/04411, an analysis of the DNA sequence for the B31 strain shows that the OspA is encoded by an open reading frame of 819 nucleotides starting at position 151 of the DNA sequence and terminating at position 970 of the DNA sequence (see Figure 1 therein). The first sixteen amino acid residues of OspA constitute a hydrophobic signal sequence of OspA. The primary translation product of the full length *B. burgdorferi* gene contains a hydrophobic N-terminal signal sequence which is a substrate for the attachment of a diacyl glycerol to the sulfhydryl side chain of the adjacent cysteine residue. Following this attachment, cleavage by signal peptidase II and the attachment of a third fatty acid to the N-terminus occurs. The complete lipid moiety is termed Pam₃Cys. It has been shown that lipidation of OspA is necessary for immunogenicity, since OspA lipoprotein with an N-terminal Pam₃Cys moiety stimulated a strong antibody response, while OspA lacking the attached lipid did not induce any detectable antibodies [Erdile et al., Infect. Immun., (1993), 61:81-90].

[0010] Published international patent application WO 91/09870 (Mikrogen Molekularbiologische Entwicklungs-GmbH) describes the DNA sequence of the *ospC* gene of *B. burgdorferi* strain Pko and the OspC (termed pC in this reference) protein encoded thereby of 22 kDa molecular weight. This sequence reveals that OspC is a lipoprotein that employs a signal sequence similar to that used for OspA. Based on the findings regarding OspA, one might expect that lipidation of recombinant OspC would be useful to enhance its immunogenicity; but, as discussed below, the applicants experienced difficulties in obtaining detectable expression of recombinant OspC.

[0011] U.S. Pat. No. 4,624,926 to Inouye relates to plasmid cloning vectors, including a DNA sequence coding for a desired polypeptide linked with one or more functional fragments derived from an outer membrane lipoprotein gene of a gram negative bacterium. The polypeptide expressed by the transformed *E. coli* host cells comprises the signal peptide of the lipoprotein, followed by the first eight amino acid residues of the lipoprotein, which in turn are followed by the amino acid sequence of the desired protein. The signal peptide may then be translocated naturally across the cytoplasmic membrane and the first eight amino acids of the lipoprotein may then be processed further and inserted into the outer membrane of the cell in a manner analogous to the normal insertion of the lipoprotein into the outer membrane. Immunogenicity of the expressed proteins was not demonstrated. Moreover, Inouye was not at all concerned with recombinant lipidation, particularly to enhance immunogenicity.

[0012] Published international patent application WO91/09952 describes plasmids for expressing lipidated proteins. Such plasmids involve a DNA sequence encoding a lipoprotein signal peptide linked to the codons for one of the β -turn tetrapeptides QANY or IEGR, which in turn is linked to the DNA sequence encoding the desired protein. Again, immunogenicity of the expressed proteins was not demonstrated.

[0013] *Streptococcus pneumoniae* causes more fatal infections world-wide than almost any other pathogen. In the U.S.A., deaths caused by *S. pneumoniae* rival in numbers those caused by AIDS. Most fatal pneumococcal infections in the U.S.A. occur in individuals over 65 years of age, in whom *S. pneumoniae* is the most common cause of community-acquired pneumonia. In the developed world, most pneumococcal deaths occur in the elderly, or in immunodeficient patients including those with sickle cell disease. In the less-developed areas of the world, pneumococcal infection is one of the largest causes of death among children less than 5 years of age. The increase in the frequency of multiple antibiotic resistance among pneumococci and the prohibitive cost of drug treatment in poor countries make the present prospect for control of pneumococcal disease problematical.

[0014] The reservoir of pneumococci that infect man is maintained primarily via nasopharyngeal human carriage. Humans acquire pneumococci first through aerosols or by direct contact. Pneumococci first colonize the upper airways and can remain in nasal mucosa for weeks or months. As many as 50% or more of young children and the elderly are colonized. In most cases, this colonization results in no apparent infection. In some individuals, however, the organism carried in the nasopharynx can give rise to symptomatic sinusitis or middle ear infection. If pneumococci are aspirated into the lung, especially with food particles or mucus, they can cause pneumonia. Infections at these sites generally shed some pneumococci into the blood where they can lead to sepsis, especially if they continue to be shed in large numbers from the original focus of infection. Pneumococci in the blood can reach the brain where they can cause meningitis. Although pneumococcal meningitis is less common than other infections caused by these bacteria, it is particularly devastating; some 10% of patients die and greater than 50% of the remainder have life-long neurological sequelae.

[0015] In elderly adults, the present 23-valent capsular polysaccharide vaccine is about 60% effective against invasive

pneumococcal disease with strains of the capsular types included in the vaccine. The 23-valent vaccine is not effective in children less than 2 years of age because of their inability to make adequate responses to most polysaccharides. Improved vaccines that can protect children and adults against invasive infections with pneumococci would help reduce some of the most deleterious aspects of this disease.

[0016] The *S. pneumoniae* cell surface protein PspA has been demonstrated to be a virulence factor and a protective antigen. In published international patent application WO 92/14488, there are described the DNA sequences for the *pspA* gene from *S. pneumoniae* Rx1, the production of a truncated form of PspA by genetic engineering, and the demonstration that such truncated form of PspA confers protection in mice to challenge with live pneumococci.

[0017] In an effort to develop a vaccine or immunogenic composition based on PspA, PspA has been recombinantly expressed in *E. coli*. It has been found that in order to efficiently express PspA, it is useful to truncate the mature PspA molecule of the Rx1 strain from its normal length of 589 amino acids to that of 314 amino acids comprising amino acids 1 to 314. This region of the PspA molecule contains most, if not all, of the protective epitopes of PspA. However, immunogenicity and protection studies in mice have demonstrated that the truncated recombinant form of PspA is not immunogenic in naive mice. Thus, it would be useful to improve the immunogenicity of recombinant PspA and fragments thereof.

Many bacterial and viral pathogens, such as *S. pneumoniae* and *Helicobacter pylori*, and HIV, herpes and papilloma viruses gain entry through mucosal surfaces. The principal determinant of specific immunity at mucosal surfaces is secretory IgA (S-IgA) which is physiologically and functionally separate from the components of the circulatory immune system. Mucosal S-IgA responses are predominantly generated by the common mucosal immune system (CMIS) [Mestecky, J. Clin. Immunol. (1987), 7:265-276], in which immunogens are taken up by specialized lympho-epithelial structures collectively referred to as mucosa-associated lymphoid tissue (MALT). The term common mucosal immune system refers to the fact that immunization at any mucosal site can elicit an immune response at all other mucosal sites. Thus, immunization in the gut can elicit mucosal immunity in the upper airways and vice versa. Further, it is important to note that oral immunization can induce an antigen-specific IgG response in the systemic compartment in addition to mucosal IgA antibodies [McGhee, J. R, et al., (1993), Infect. Agents and Disease 2:55-73].

[0018] Most soluble and non-replicating antigens are poor mucosal immunogens, especially by the peroral route, probably because they are degraded by digestive enzymes and have little or no tropism for the gut associated lymphoid tissue (GALT). Thus, a method for producing effective mucosal immunogens, and vaccines and immunogenic compositions containing them, would be desirable.

[0019] Of particular interest is *H. pylori*, the spiral bacterium which selectively colonizes human gastric mucin-secreting cells and is the causative agent in most cases of nonexosive gastritis in humans. Recent research indicates that *H. pylori*, which has a high urease activity, is responsible for most peptic ulcers as well as many gastric cancers. Many studies have suggested that urease, a complex of the products of the *ureA* and *ureB* genes, may be a protective antigen. However, until now it has not been known how to produce a sufficient mucosal immune response to urease.

[0020] Antigens, such as OspC, PspA, UreA, UreB or immunogenic fragments thereof, stimulate an immune response when administered to a host. Such antigens, especially when recombinantly produced, may elicit a stronger response when administered in conjunction with an adjuvant. Currently, alum is the only adjuvant licensed for human use, although hundreds of experimental adjuvants such as cholera toxin B are being tested. However, these adjuvants have deficiencies. For instance, while cholera toxin B is not toxic in the sense of causing cholera, there is general unease about administering a toxin associated with a disease as harmful as cholera, especially if there is even the most remote chance of minor impurity.

[0021] Thus, it would be desirable to enhance the immunogenicity of antigens, by methods other than the use of an adjuvant, especially in monovalent preparations; and, in multivalent preparations, to have the ability to employ such a means for enhanced immunogenicity with an adjuvant, so as to obtain an even greater immunological response.

[0022] As to expression of recombinant proteins, it is expected that the skilled artisan is familiar with the various vector systems available for such expression, e.g., bacteria such as *E. coli* and the like.

[0023] It is believed that heretofore the art has not taught or suggested expression of a recombinant OspC lipoprotein of a *Borrelia* species wherein the lipidation thereof is from expression of a first nucleic acid sequence encoding an OspA leader sequence of a *Borrelia* species, the protein thereof is from expression of a second nucleic acid sequence, the first and second sequences, which do not naturally occur together, being contiguous, or genes containing such sequences; or vectors containing such sequences; or methods for such expression; or such recombinant lipoproteins; or compositions containing such recombinant lipoproteins; or methods for using such compositions; or methods for enhancing the immunogenicity of a protein by lipidation from a nucleic acid sequence not naturally occurring with the nucleic acid sequence encoding the protein portion of the lipoprotein.

OBJECTS AND SUMMARY OF THE INVENTION

[0024] It is an object of the invention to provide a recombinant OspC lipoprotein of a *Borrelia* species wherein the lipidation

thereof is from expression of a first nucleic acid sequence encoding an OspA leader sequence of a *Borrelia* species and the protein portion thereof is from expression of a second nucleic acid sequence and the first and second sequences do not naturally occur together.

[0025] It is another object of the invention to provide expression of genes and/or sequences encoding such a recombinant lipoprotein, vectors therefor and methods for effecting such expression.

[0026] It is a further object of the invention to provide immunogenic compositions, including vaccines, containing the recombinant OspC lipoproteins of a *Borrelia* species and/or vectors for expression thereof.

[0027] It has surprisingly been found that an immunogenic recombinant lipidated OspC protein of a *Borrelia* species, can be expressed from a vector system, preferably *E. coli*, without the toxicity to the vector system evident when the native lipoprotein signal sequence encoding region is present. This result has been achieved by replacing the nucleotide sequence encoding the native leader or signal sequence of a lipoprotein with the nucleotide sequence encoding a leader or signal of OspA-lipoprotein of a *Borrelia* species. These recombinant lipidated proteins have been shown to elicit an immune response, including a mucosal immune response.

[0028] It is surprising that fusion of DNA encoding an OspA lipoprotein leader sequence of a *Borrelia* species directly to the DNA encoding an OspC protein of a *Borrelia* species, without any intervening nucleotide sequences, can lead to expression of an immunogenic recombinant lipoprotein in significant quantities without the toxicity evident with the native leader sequence, because previous attempts to express recombinant lipidated proteins have been unsuccessful. For example, Fuchs et al. report that recombinantly formed OspC (referred to as pC in this reference) with its native leader protein was only weakly expressed in *E. coli* [Mol. Microbiology (1992) 6 (4) :503-509]. Applicants, in addition to Fuchs, attempted to obtain lipidated recombinant OspC by expression of the OspC-encoding sequence in *E. coli* using the pET vector system described in the aforementioned WO 92/14488 for the expression of OspA and using the pDS12 plasmid systems described. However, OspC was barely detectable by immunoblotting of cell extracts using these systems to express OspC.

[0029] Further, as discussed supra, it was believed that an additional nucleotide sequence, preferably one encoding a peptide sequence forming a β -turn, was necessary for expression of recombinant lipoproteins, and the immunogenicity of recombinant lipoproteins previously expressed had not been demonstrated.

[0030] The procedure of the present invention, therefore, enables large quantities of pure recombinant, immunogenic lipidated OspC proteins of a *Borrelia* species, to be obtained, which has not heretofore been possible. The recombinantly-formed lipidated proteins provided herein are significantly more immunogenic than a non-lipidated recombinant analog.

[0031] The present invention, it is believed, represents the first instance of effecting expression of a heterologous lipidated OsPC protein of a *Borrelia* species using the non-native, OspA leader sequence. The invention, therefore, includes the use of a non-native, OspA leader sequence to express OspC proteins heterologous to the leader sequence.

[0032] Accordingly, in one embodiment, the present invention provides an isolated hybrid nucleic acid molecule, preferably DNA, comprising a first nucleic acid sequence encoding the signal sequence of an OspA protein of a *Borrelia* species, coupled in translational open reading frame relationship with a second nucleic acid sequence encoding a mature OspC lipoprotein of a *Borrelia* species heterologous to the signal sequence.

[0033] More preferably, the first and second sequences are contiguous when the mature protein is naturally lipidated, and separated by one codon coding for one amino acid, preferably cysteine, when the mature protein is not naturally lipidated.

[0034] The mature OspC lipoprotein encoded by the second nucleic acid sequence is preferably an antigenic lipoprotein, and more preferably a strain of *B. burgdorferi*, more preferably a strain of *B. burgdorferi* selected from the OspC sub-type families.

[0035] The hybrid gene provided herein may be assembled into an expression vector, preferably under the control of a suitable promoter for expression of the mature OspC lipoprotein of a *Borrelia* species, in accordance with a further aspect of the invention, which, in a suitable host organism, such as *E. coli*, causes initial translation of a chimeric molecule comprising the OspA leader sequence of a *Borrelia* species and the desired heterologous OspC protein in lipidated form, followed by cleavage of the chimeric molecule by signal peptidase II and attachment of lipid moieties to the new terminus of the protein, whereby the mature OspC lipoprotein is expressed in the host organism.

[0036] The present invention provides, for the first time, a hybrid nucleic acid molecule which permits the production of recombinant lipidated OspC protein of a *Borrelia* species.

[0037] Accordingly, in a further aspect of this invention, there is provided a hybrid nucleic acid molecule, comprising a first nucleic acid sequence encoding an OspC lipoprotein of a *Borrelia* species, more preferably a strain of *B. burgdorferi*, still more preferably a strain of *B. burgdorferi* selected from the OspC sub-type families; and a second nucleic acid sequence encoding an OspA signal sequence of an expressed protein of a *Borrelia* species coupled in translational open reading frame relationship with said first nucleic acid sequence.

[0038] As described above, the hybrid gene can be assembled into an expression vector under the control of a suitable promoter for expression of the OspC lipoprotein, which, in a suitable host organism, such as *E. coli*, causes expression of the OspC lipoprotein from the host organism.

[0039] It has also surprisingly been found that enhanced immunogenicity can be obtained by a recombinant OspC lipoprotein of a *Borrelia* species when the OspC lipoprotein is expressed by a hybrid or chimeric gene comprising a first nucleic acid sequence encoding an OspA leader or signal sequence of a *Borrelia* species and a second nucleic acid sequence encoding the protein portion of the OspC lipoprotein, wherein the first and second sequences do not naturally occur together.

[0040] Accordingly, the present invention also provides a recombinant OspC lipoprotein of a *Borrelia* species expressed by a hybrid or chimeric gene comprising a first nucleic acid sequence encoding an OspA leader or signal sequence of a *Borrelia* species contiguous with a second nucleic acid sequence encoding a protein portion of the OspC lipoprotein, and the first and second sequences do not naturally occur together. The second sequence can encode a an OspC antigen. The first and second sequences can be present in a gene; and the gene and/or the first and second sequences can be in a suitable vector for expression.

[0041] The vector can be a nucleic acid in the form of, e.g., plasmids, bacteriophages and integrated DNA, in a bacteria, most preferably one used for expression, e.g. *E. coli*, *Bacillus subtilis*, *Salmonella*, *Staphylococcus*, *Streptococcus*, etc., or one used as a live vector, e.g. *Lactobacillus*, *Mycobacterium*, *Salmonella*, *Streptococcus*, etc. When an expression host is used the recombinant lipoprotein can be obtained by harvesting product expressed *in vitro*; e.g., by isolating the recombinant lipoprotein from a bacterial extract. The gene can preferably be under the control of and therefore operably linked to a suitable promoter; and the promoter can either be endogenous to the vector, or be inserted into the vector with the gene.

[0042] The invention further provides vectors containing the nucleic acid encoding the recombinant OspC lipoprotein and methods for obtaining the recombinant OspC lipoproteins and methods for preparing the vector.

[0043] As mentioned, the recombinant lipoprotein can have enhanced immunogenicity. Thus, additional embodiments of the invention provide immunogenic or vaccine compositions for inducing an immunological response, comprising the isolated recombinant OspC lipoprotein, or a suitable vector for *in vivo* expression thereof in a non-animal host, or both, and a suitable carrier, and the use of said compositions in the preparation of a medicament for the prophylaxis or treatment of Lyme disease.

[0044] Documents cited in this disclosure, including the above-referenced applications, provide typical additional ingredients for such compositions, such that undue experimentation is not required by the skilled artisan to formulate a composition from this disclosure. Such compositions should preferably contain a quantity of the recombinant OspC lipoprotein of a *Borrelia* species or vector expressing such sufficient to elicit a suitable response. Such a quantity of recombinant OspC lipoprotein or vector can be based upon known amounts of antigens administered. For instance, if there is a known amount for administration of an antigen corresponding to the second sequence expressed for the inventive recombinant OspC lipoprotein, the quantity of recombinant lipoprotein can be scaled to about that known amount, and the amount of vector can be such as to produce a sufficient number of colony forming units (cfu) so as to result in *in vivo* expression in a non-animal host of the recombinant ospC lipoprotein in about that known amount. Likewise, the quantity of recombinant lipoprotein can be based upon amounts of antigen administered to animals in the examples below and in the documents cited herein, without undue experimentation.

[0045] The present invention also includes, in other aspects, processes for the production of a recombinant OspC lipoprotein of a *Borrelia* species, by assembly of an expression vector, expression of the lipoprotein from a non-animal host organism containing the expression vector, and optionally isolating and/or purifying the expressed OspC lipoprotein. The isolating/purifying can be so as to obtain recombinant lipoprotein free from impurities such as lipopolysaccharides and other bacterial proteins. The present invention further includes immunogenic compositions for inducing an immunological response, such as vaccines, containing the recombinant lipoprotein as well as the use of said compositions in the preparation of a medicament for the prophylaxis or treatment of Lyme disease.

BRIEF DESCRIPTION OF DRAWINGS

[0046] In the following detailed description, reference is made to the accompanying drawings, wherein:

Figure 1 is a schematic representation of a procedure for assembling plasmid pLF100;

Figure 2 is a schematic representation of a procedure for assembling plasmid vectors pPko9a (strain Pko) and pB319a (strain B31) ;

Figure 3 is a schematic representation of the procedure employed for the isolation and purification of lipidated OspC; Figure 4 is a schematic representation of the procedure employed for the isolation and purification of non-lipidated OspC for comparative purposes;

Figure 5 shows an SDS-PAGE analysis of lipidated OspC produced herein at various stages of the purification procedure illustrated schematically in Figure 3;

Figure 6 shows an SDS-PAGE analysis of non-lipidated OspC produced herein at various stages of the purification procedure as described in WO 91/09870;

Figure 7 is a graphical representation of the immune response of mice immunized with OspC formulations containing antigen from two OspC sub-types as measured in an anti-OspC ELISA assay;

Figure 8 is a graphical representation of the immune response of mice immunized with a two sub-type OspC formulation that contains alum adjuvant as measured in an anti-OspC ELISA assay;

Figure 9 is a schematic representation of a procedure for assembling plasmid vector pPA321-L;

Figure 10 is a schematic representation of a procedure for assembling plasmid vector pPA321-NL;

Figure 11 is a schematic representation of the procedure employed for the isolation and purification of lipidated PspA;

Figure 12 is a schematic representation of the procedure employed for the isolation and purification of non-lipidated PspA for comparative purposes;

Figure 13 shows an SDS-PAGE analysis of lipidated PspA produced herein at various states of the expression and host cell fractionation procedure illustrated schematically in Figure 11;

Figure 14 shows an SDS-PAGE analysis of non-lipidated PspA produced herein at various stages of the expression and host cell fractionation procedure illustrated schematically in Figure 12; and

Figure 15 shows an SDS-PAGE analysis of the PspA column chromatography results illustrated schematically in Figures 11 and 12.

DETAILED DESCRIPTION OF INVENTION

[0047] As noted above, the present invention is concerned with the use of a nucleic acid sequence encoding the OspA signal sequence to express a lipidated OspC protein of a *Borrelia* species heterologous to OspA protein, and to the use of a nucleic acid sequence encoding the OspA signal sequence of a *Borrelia* species of a protein heterologous to the OspC protein to be expressed, to express the lipidated OspC protein of a *Borrelia* species.

[0048] The leader amino acid sequence and encoding DNA sequence for the ACA strain of *B. burgdorferi* are as follows:

M K K Y L L G I G L I L A L I A C

(SEQ ID NO: 1)

ATG AAA AAA TAT TTA TTG GGA ATA GGT CTA ATA TTA GCC TTA ATA GCA TGC

(SEQ ID NO: 2)

The corresponding leader amino acid sequences and encoding DNA sequences for the OspA of other strains of *B. burgdorferi* are known in the art and may be employed in the present invention from this disclosure, without any undue experimentation.

[0049] A hybrid gene molecule is assembled comprising the OspA leader encoding sequence and the gene encoding the heterologous protein to be expressed, preferably the *ospC* or *pspA* gene, arranged in translational reading-frame relationship with the *ospA* gene fragment.

[0050] For production of the lipidated protein, the appropriate hybrid gene molecule can be incorporated into a suitable expression vector and the resulting plasmid incorporated into an expression strain of *E. coli* or other suitable host organism. The vector can also be a bacteriophage or integrated DNA.

[0051] The lipidated protein is expressed by the cells during growth of the host organism. The lipidated protein may be recovered from the host organism in purified form by any convenient procedure which separates the lipidated protein in undenatured form. One schematic of a procedure in accordance with this aspect of the invention is shown in Figure 3.

[0052] Following cell growth and induction of protein, the cells are subjected to freeze-thaw lysis and DNase I treatment. The lysate is treated with a detergent which is selective for solubilization of the recombinant lipidated protein, in preference to the other bacterial proteins in the lysate. While the present invention preferably utilizes polyethylene glycol *tert*-octyl-phenyl ether having the formula $t\text{-Oct-C}_6\text{H}_4(\text{OCH}_2\text{CH}_2)_x\text{OH}$ wherein $x=7-8$ as the detergent (commercially available as, and hereinafter referred to as, TRITON™ X-114), other materials may be used exhibiting a similar selective solubility for the lipidation protein as well as the phase separation property under mild conditions, as discussed below.

[0053] Following addition of the TRITON™ X-114, the mixture is warmed to a mild temperature elevation of preferably about 35°C to 40°C, at which time the solution becomes cloudy as phase separation occurs. The purification procedure for such phase separation should occur under conditions to avoid any substantial denaturing or any other substantial impairment of the immunological properties of the recombinant lipoprotein.

[0054] Centrifugation of the cloudy mixture results in separation of the mixture into three phases, namely a detergent phase containing about 50% or more of the recombinant lipidate protein and a small amount (approximately 5 wt%) of other proteins, an aqueous phase containing the balance of the other proteins, and a solid pellet of cell residue. The detergent phase is separated from the aqueous phase and the solid pellet for further processing.

[0055] Final purification of the protein preferably is effected by processing of the detergent phase to provide a recombinant lipidated protein having a purity of at least about 80 wt%, and which is substantially free from other contaminants such as bacterial proteins, and lipopolysaccharides (LPS), and which has endotoxin levels compatible with human

administration.

[0056] Such purification is conveniently effected by column chromatography. Such chromatographic purification may include a first chromatographic purification using a first chromatographic column having the pH, ionic strength and hydrophobicity to bind bacterial proteins, but not the recombinant lipidated protein.

[0057] Such first chromatographic purification may be effected by loading the detergent phase onto the first chromatographic column and the flow-through, which contains the purified lipidated protein, is collected. The bound fraction contains substantially all the bacterial protein impurities from the detergent phase. The chromatography medium used for such first purification operation may be a DEAE-Sepacel or DEAE-Sepharose column.

[0058] The flow-through from the first chromatographic purification operation may be subjected to further purification on a second chromatographic column. The flow-through is loaded onto the column having the pH, ionic strength and hydrophobicity which will selectively bind the recombinant lipidated protein to the second chromatographic column, while bacterial contaminants and LPS pass through the column. The chromatography medium for the second chromatographic column may be S-Sepharose.

[0059] Preferably the recombinant *OspC* lipoprotein of a *Borrelia* species is purified to 80% purity or to greater than 80% purity, e.g., 85-90% or even 90-95% or greater than 95% purity. The lipidated proteinaceous material can then be formulated into immunogenic compositions, preferably vaccines.

[0060] The vaccine or immunogenic composition elicits an immune response in a host subject which produces an immunological response, such as antibodies which may be opsonizing or bactericidal. Should a subject immunized with a recombinant lipoprotein of the invention then be challenged, such immunological response can inactivate the challenge organism. Furthermore, opsonizing or bactericidal antibodies may also provide protection by alternative mechanisms.

[0061] Immunogenic compositions including vaccines can be prepared as injectables, as liquid solutions or emulsions, or as formulating for oral, nasal or other orifice administration e.g., vaginal, rectal, etc. Oral formulations can be liquid solutions, emulsions and the like, e.g., elixirs, or solid preparations, e.g., tablets, caplets, capsules, pills, liquid-filled-capsules, gelatin and the like. Nasal preparations can be liquid and can be administered via aerosol, squeeze spray or pump spray dispensers. Documents cited herein provide exemplary formulation types and ingredients therefor, including the applications cited above.

[0062] The immunogens can be mixed with pharmaceutically acceptable excipients which are compatible with the immunogens. Such excipients may include water, saline, dextrose, glycerol, ethanol, and combinations thereof. The immunogenic compositions and vaccines may further contain auxiliary substances, such as wetting or emulsifying agents, pH buffering agents, or adjuvants to enhance the effectiveness thereof. Immunogenic compositions and vaccines may be administered parenterally, by injection subcutaneously or intramuscularly. The immunogenic preparations and vaccines are administered in a manner compatible with the dosage formulation, and in such amount as will be therapeutically effective, immunogenic or protective. The quantity to be administered depends on the subject to be treated, including, for example, the capacity of the immune system of the individual to synthesize antibodies, and, if needed, to produce a cell-mediated immune response. Precise amounts of active ingredient required to be administered depend on the judgment of the practitioner, taking into account such factors as the age, weight, sex, condition of the host or patient to whom there is to be administration. However, suitable dosage ranges are readily determinable by one skilled in the art and may be of the order of micrograms of the immunogens. Suitable regimes for initial administration and booster doses are also variable, but may include an initial administration followed by subsequent administrations. The dosage may also depend on the route of administration and will vary according to the size of the host.

[0063] The concentration of the immunogens in a composition comprising the *OspC* lipoprotein according to the invention for inducing an immunological response is in general about 1 to about 95%. A vaccine or immunogenic composition which contains antigenic material of only one pathogen is a monovalent vaccine. Vaccines or compositions comprising the *OspC* lipoprotein of the present invention for inducing an immunological response, which are multivalent or which contain antigenic material of several pathogens (also known as combined vaccines or combined immunogenic compositions) also belong to the present invention. Such combined vaccines or immunogenic compositions contain, for example, material from various pathogens or from various strains of the same pathogen, or from combinations of various pathogens.

[0064] Immunostimulatory agents or adjuvants have been used for many years to improve the host immune responses to, for example, vaccines or immunogenic compositions. Intrinsic adjuvants, such as lipopolysaccharides, normally are the components of the killed or attenuated bacteria used as vaccines or immunogenic compositions. Extrinsic adjuvants are immunomodulators which are typically non-covalently linked to antigens and are formulated to enhance the host immune responses. Some of these adjuvants are toxic, however, and can cause undesirable side-effects, making them unsuitable for use in humans and many animals. Indeed, only alum is routinely used as an adjuvant in human and veterinary vaccines.

[0065] In view of the difficulties associated with the use of adjuvants, it is thus an advantage of the present invention that the recombinant lipidated *OspC* proteins of a *Borrelia* species are the most immunogenic forms, and are capable of eliciting immune responses both without any adjuvant and with alum.

[0066] The following examples illustrate but do not limit the scope of the invention disclosed in this specification.

EXAMPLES

Example 1

Construction of a Vector Containing a Gene Encoding the OspA Leader Sequence

[0067] Plasmid pBluescript KS+ (Stratagene) was digested with *Xba*I and *Bam*HI and ligated with a 900 bp *Xba*I-*Bam*HI DNA fragment containing the complete coding region of *B. burgdorferi* strain ACA1 *ospA* gene, to form a lipoprotein fusion vector pLF100. This procedure is shown schematically in Figure 1.

[0068] The vector pLF100, has been deposited with the American Type Culture Collection 10801 University Blvd., Manassas, Va. 20110-2209 on February 2, 1995 under Accession No. 69750. This deposit was made under the terms of the Budapest Treaty.

Example 2

Construction of a Expression Vector Containing a Hybrid *ospA-ospC* Gene

[0069] Specifically designed oligonucleotide primers were used in a polymerase chain reaction (PCR) to amplify the portion of the *ospC* gene downstream from the cysteine-encoding codon terminating the signal-peptide recognition-encoding sequence to the C-terminal end of the coding region from the Pko and B31 strains of *B. burgdorferi*.

[0070] The 5'-end primer had the nucleotide sequences respectively for the Pko and B31 strains:

5'-GGC GCG CAT GCA ATA ATT CAG GGA AAG G-3' (Pko) (SEQ ID NO: 3)

5'-GGC GCG CAT GCA ATA ATT CAG GGA AAG A-3' (B31) (SEQ ID NO: 4)

while the 3'-end primer had the nucleotide sequence:

5'-CGC GGA TCC TTA AGG TTT TTT TGG-3' (B31 & Pko) (SEQ ID NO: 5)

[0071] The PCR amplification was effected in a DNA Thermal Cycler (Perkins-Elmer Cetus) for 25 cycles with denaturation for 30 secs at 94°C., annealing at 37°C for 1 minute and extension at 72°C. for minute. A final extension was effected at 72°C. for 5 minutes at the completion of the cycles. The product was purified using a Gene Clean II kit (B10 101) and the purified material was digested with SphI and BamHI. This procedure introduced a silent mutation in the Pko *ospC* gene which changes the codon for amino acid 60 of the mature protein from ATT to ATA.

[0072] The materials produced for the Pko and B31 *B. burgdorferi* strains were handled identically from this point on and hence only the further handling of the Pko strain OspC material is described.

[0073] The plasmid pLF100 (Example 1) was digested with SphI and BamHI and the amplified Pko sequence was ligated into the plasmid to form plasmid pPko 100 (pB31 100 for the B31 strain) containing a hybrid *ospA/ospC* gene. The hybrid gene was excised from plasmid pPko 100 by digestion with NdeI and BamHI and cloned into the NdeI and BamHI sites of the plasmid vector pET9 to place the *ospA/ospC* hybrid gene under control of a T7 promoter and efficient translation initiation signals from bacteriophage T7, as seen in Figure 2. The resulting plasmid is designated pPko9a (pB319a for the B31 strain).

Example 3

Expression and Purification of Lipidated OspC

[0074] Plasmid pPko9a, prepared as described in Example 2, was used to transform *E. coli* strains BL21(DE3)(pLysS) and HMS174(DE3)(pLysS). The transformed *E. coli* was inoculated in to LB media with 30 µg/ml kanamycin sulfate and 25 µg/ml of chloramphenicol at a rate of 12 ml of culture for every liter prepped. The culture was grown overnight in a flask shaker at 37°C.

[0075] The next morning, 10 ml of overnight culture medium was transferred to 1 L of LB media containing 30 µg/ml of kanamycin sulfate and the culture was grown in a flask shaker at about 37°C to a level of OD₆₀₀=0.6-1.0 (although growth up to OD₆₀₀=1.5 can be effected), in approximately 3-5 hours.

[0076] To the culture medium was added isopropylthiogalactoside (IPTG) to a final concentration of 0.5 mM and the culture medium was grown for a further two hours at about 30°C. The cultures were harvested and samples analyzed

on Coomassie stained SDS-PAGE gels (Figure 5). The culture medium was cooled to about 4°C and centrifuged at 10,000 xG for 10 minutes. The supernatant was discarded while the cell pellet was collected. Purified lipidated OspC was recovered from the pellet by effecting the procedure shown schematically in Figure 3 and described below.

[0077] The cell pellet first was resuspended in 1/10 the volume of PBS. The cell suspension was frozen and stored at -20°C or below, if desired. Following freezing of the cell suspension, the cells were thawed to room temperature (about 20° to 25°C) which causes the cells to lyse. DNase I was added to the thawed material to a concentration of 1 µg/ml and the mixture was incubated for 30 minutes at room temperature, which resulted in a decrease in the viscosity of the material.

[0078] The incubated material was chilled on ice to a temperature below 10°C and TRITON™ X-114 was added as a 10 wt% stock solution, to a final concentration of 0.3 to 1 wt%. The mixture was kept on ice for 20 minutes. The chilled mixture next was heated to about 37°C and held at that temperature for 10 minutes.

[0079] The solution turned very cloudy as phase separation occurred. The cloudy mixture then was centrifuged at about 20°C for 10 minutes at 12,000 xG, which caused separation of the mixture into a lower detergent phase, an upper clear aqueous phase and a solid pellet. Analysis of the phases fractionated by SDS-PAGE (Figure 5) revealed that the OspC partitioned into the detergent phase, showing that it is in lipidated form. The detergent phase was separated from the other two phases and cooled to 4°C., without disturbing the pellet.

[0080] Buffer A, namely 50 mM Tris pH 7.5, 2 mM EDTA and 10 mM NaCl and 0.3% polyethylene glycol tert-octylphenyl ether having the formula t-Oct-C₆H₄-(OCH₂CH₂)_xOH wherein x=9-10 as the detergent (commercially available as, and hereinafter referred to as, TRITON™ X-100), was added to the cooled detergent phase to reconstitute back to 1/3 the original volume. The resulting solution may be frozen and stored for later processing as described below or may be immediately subjected to such processing.

[0081] A DEAE-Sepharose CL-6B column was prepared in a volume of 1 ml/10 ml of detergent phase and was washed with 2 volumes of Buffer C, namely 50 mM Tris pH 7.5, 2 mM EDTA, 1 M NaCl, 0.3 wt% TRITON™ X-100, and then with 4 volumes of Buffer B, namely 50 mM Tris pH 7.5, 2 mM EDTA, 0.3 wt% TRITON™ X-100.

[0082] The detergent phase then was loaded onto the column and the flow-through containing the OspC, was collected. The column was washed with 2 volumes of Buffer B and the flow-through again was collected. The combined flow-through was an aqueous solution of purified OspC, which may be frozen for storage.

[0083] The column may be freed from bacterial proteins for reuse by eluting with 4 volumes of Buffer C.

[0084] Further and final purification of the flow-through from the DEAE-Sepharose column was effected by chromatography on S-Sepharose Fast Flow. The flow-through from the DEAE-Sepharose column first was acidified to pH 4.3 by the addition of 1 M citric acid and the acidified material was loaded onto the S-Sepharose column. The S-Sepharose Fast Flow column had been washed with 3 column volumes of Buffer A and then with 5 column volumes of Buffer A made up to pH 4.3. The OspC binds to the column. The loaded column was washed with 4 column volumes of pH 4.3 Buffer A followed by 4 column volumes of pH 5.5 Buffer A.

[0085] Highly-purified OspC was eluted from the column using Buffer A, adjusted to pH 6.0 with 1N HCl. A schematic of the purification process described in this Example is shown in Figure 3.

[0086] The aqueous solution of highly purified lipidated OspC obtained by the chromatography procedures was analyzed by Coomassie stained gels (Figure 5), and confirmed to be OspC in highly purified form by immunoblot analysis using rabbit anti-OspC polyclonal antiserum. The purity of the product was estimated to be greater than 80%.

[0087] By this procedure, about 2 to 4 mg of pure OspC was recovered from a 1 liter culture of the BL21 host and about 1 to 2 mg of pure OspC was recovered from a 1 liter culture of the HMS 174 host.

Example 4

Expression and Purification of Non-lipidated OspC

[0088] *E. coli* JM 109 transformants containing plasmid vector containing chromosomal gene fragment encoding non-lipidated OspC were prepared and grown as described in WO 91/09870. The cultures were harvested, the culture medium centrifuged at 10,000 xG for 10 minutes at 4°C., the supernatant discarded and the pellet collected.

[0089] The cell pellet was first resuspended in lysis buffer A, namely 50mM Tris-HCl pH 8.0, 2mM EDTA, 0.1mM DTT, 5% glycerol and 0.4mg/ml lysozyme, and the suspension stirred for 20 minutes at room temperature. TRITON™ X-100 then was added to the cell suspension to a concentration of 1 wt%, DNase I was added to a concentration of 1 µg/ml, and the suspension stirred at room temperature for a further 20 minutes to effect cell lysis. Sodium chloride next was added to the cell suspension to a concentration of 1M and the suspension again stirred at 4°C for a further 20 minutes. The suspension then was centrifuged at 20,000 x G for 30 minutes, the resultant supernatant separated from the pellet and the pellet was discarded.

[0090] The separated supernatant was dialyzed against a buffer comprising 50 mM Tris pH 8, 2 mM EDTA. The supernatant next was loaded onto a DEAE-Sepharose CL-6B column and the non-lipidated OspC was collected in the

column flow-through. The flow-through was dialyzed against a 0.1 M phosphate buffer, pH 6.0.

[0091] The dialyzed flow-through next was bound to a S-Sepharose fast flow column equilibrated with 0.1M phosphate buffer, pH 6.0. Purified non-lipidated OspC then was eluted from the S-Sepharose column using the dialysis buffer with 0.15 M NaCl added. A schematic of the purification process is shown in Figure 4.

[0092] The aqueous solution of highly purified non-lipidated OspC was analyzed by Coomassie stained gels (Figure 6). The purity of the product was estimated to be greater than 80%.

Example 5

Immunogenicity of Recombinant Lipidated OspC

[0093] Purified recombinant lipidated OspC, prepared as described in Example 3, was tested for immunogenicity in mice and compared to that from non-lipidated OspC prepared as described in Example 4. For this study, 4 to 8 week old female C3H/He mice were immunized on day 0 and boosted on day 21 and 42. All animals were given 1 μ g each of OspC expressed from the B31 and Pko genes per dose. Both lipidated and non-lipidated forms of the antigen were tested. Formulations were tested with and without alum as an adjuvant.

[0094] Representative animals were exsanguinated on days 21, 42, 63 and 91. ELISA testing was performed on these sera using purified non-lipidated OspC as the coating antigen.

[0095] The test results from mice immunized with unadjuvanted antigen (Figure 7) show that only animals immunized with the lipidated antigen make a detectable ELISA response. However, the immune response of animals immunized with antigens formulated on alum (Figure 8) shows that two types of antigen give comparable ELISA responses and these responses develop more rapidly.

Construction of a pET9a Expression Vector Containing a Hybrid *ospA/pspA* Gene (For background information only)

[0096] Specifically designed oligonucleotide primers were used in a PCR reaction to amplify the portion of the *pspA* gene of interest (in this case from amino acid 1 to 321) from the *S. pneumoniae* strain RX1.

[0097] The 5'-end primer had the nucleotide sequence:

5'-GGG ACA GCA TGC GAA GAA TCT CCC GTA GCC AGT-3' (PspN1)
(SEQ ID NO: 6).

[0098] The 3'-end primer had the nucleotide sequence:

5'-GAT GGA TCC TTT TGG TGC AGG AGC TGG TTT-3' (PspC370)
(SEQ ID NO: 7).

[0099] The PCR reaction was as follows: 94°C for 30 seconds to denature DNA; 42°C for one minute for annealing DNA; and 72°C for one minute for extension of DNA. This was carried out for 25 cycles, followed by a 5 minute extension at 72°C. This procedure introduced a stop codon at amino acid 315. The PCR product was purified using the Gene Clean II method (Bio101), and digested with SphI and BamHI.

[0100] The plasmid pLF100 (Example 1) was digested with SphI and BamHI and the amplified *pspA* gene was ligated to this plasmid to form the plasmid pLF321, which contained the hybrid *ospA-pspA* gene. The hybrid gene was excised from pLF321 by digestion with NdeI and BamHI and cloned into the NdeI and BamHI sites of the plasmid vector pET9a to place the *ospA-pspA* hybrid gene under the control of a T7 promoter. The resulting plasmid is called pPA321-L. This process is shown schematically in Figure 9.

Construction of a pET9a Expression Vector Containing the *pspA* Gene (For background information only)

[0101] Specifically designed oligonucleotide primers were used in a PCR reaction to amplify the portion of the *pspA* gene of interest (in this case from amino acid 1 to 321) from the *S. pneumoniae* strain RX1 using plasmid pPA321-L of Example 6.

[0102] The 5'-end primer had the nucleotide sequence:

5'-GCT CCT GCA TAT GGA AGA ATC TCC CGT AGC C-3' (PspNL-2)
(SEQ ID NO: 8)

[0103] The 3'-end primer had the nucleotide sequence:

5'-GAT GGA TCC TTT TGG TGC AGG AGC TGG TTT-3' (PspC370)
(SEQ ID NO: 7).

[0104] The PCR reaction was as follows: 94°C for 30 seconds to denature DNA; and 72°C for one minute for annealing and extension of DNA. This was carried out for 25 cycles, which was followed by a 5 minute extension at 72°C. This procedure introduced a stop codon at amino acid 315. The PCR product was purified using the Gene Clean II method (Bio 101), and digested with NdeI and BamHI. The digested PCR product was cloned into the NdeI and BamHI sites of the plasmid vector pET9a to place the *pspA* gene under the control of a T7 promoter. The resulting plasmid is called pPA321-NL. This process is shown schematically in Figure 10.

Expression and Purification of Lipidated PspA (For background information only)

[0105] Plasmid pPA321-L was used to transform *E. coli* strain BL21(DE3)pLyS. The transformed *E. coli* was inoculated into LB media containing 30 µg/ml kanamycin sulfate and 25 µg/ml chloramphenicol. The culture was grown overnight in a flask shaker at 37°C.

[0106] The following morning 50ml of overnight culture was transferred to 1 L LB media containing 30 µg/ml kanamycin sulfate and the culture was grown in a flask shaker at 37°C to a level of OD 600nm of 0.6-1.0, in approximately 3-5 hours. To the culture medium was added IPTG to a final concentration of 0.5 mM and the culture was grown for an additional two hours at 30°C. The cultures were harvested by centrifugation at 4°C at 10,000xG and the cell pellet collected. Lipidated PspA was recovered from the cell pellet.

[0107] The cell pellet was resuspended in PBS at 30 g wet cell paste per liter PBS. The cell suspension was frozen and stored at -20°C. The cells were thawed to room temperature to effect lysis. DNaseI was added to the thawed material at a final concentration of 1 µg/ml and the mixture incubated for 30 minutes at room temperature, which resulted in a decrease in viscosity of the material.

[0108] The material was then chilled in an ice bath to below 10°C. and TRITON™ X-114 was added as a 10% stock solution to a final concentration of 0.3 to 1%. The mixture was kept on ice for 20 minutes. The chilled mixture was then heated to 37°C and held at that temperature for 10 minutes. This caused the solution to become very cloudy as phase separation occurred. The mixture was then centrifuged at about 20°C for 10 minutes at 12,000xG, which caused a separation of the mixture into a lower detergent phase, an upper clear aqueous phase and a pellet. The lipidated PspA partitioned into the detergent phase. The detergent phase was separated from the other two phases, diluted 1:10 with a buffer comprising 50mM Tris, 2mM EDTA, 10mM NaCl pH 7.5, and was stored at -20°C.

[0109] A Q-Sepharose column was prepared in a volume of 1 ml per 5 ml diluted detergent phase. The column was washed with 2 column volumes of a buffer comprising 50mM Tris, 2mM EDTA, 0.3% TRITON™ X-100, 1M NaCl pH 4.0, and then equilibrated with 5 to 10 column volumes 50mM Tris, 2mM EDTA, 0.3% TRITON™ X-100, 10mM NaCl pH 4.0. The pH of the diluted detergent phase material was adjusted to 4.0, at which time a precipitation occurred. This material was passed through a 0.2 µm cellulose acetate filtering unit to remove the precipitated material. The filtered diluted detergent phase was applied to the Q-Sepharose column and the flow through (containing PA321-L) was collected. SDS-PAGE analysis of this step is shown in Figure 15. The column was washed with 1-2 column volumes of 50mM Tris, 2mM EDTA, 0.3% TRITON™ X-100, 10mM NaCl pH 4.0, and the flow through was pooled with the previous flow through fraction. The pH of the flow through pool was adjusted to 7.5. The bound material, contaminating *E. coli* proteins, was eluted from the Q-Sepharose with 2 column volumes of 50mM Tris, 2mM EDTA, 0.3% TRITON™ X-100, 1M NaCl pH 4.0. A schematic of the purification process described in this Example is shown in Figure 11.

Expression and Purification of Non-lipidated PspA (For background information only)

[0110] Plasmid pPA321-NL was used to transform *E. coli* strain BL21 (DE3)pLyS. The transformed *E. coli* was inoculated into LE media. containing 30 µg/ml kanamycin sulfate and 25 µg/ml chloramphenicol. The culture was grown overnight in a flask shaker at 37°C.

[0111] The following morning 50 ml of overnight culture was transferred to 1 L LB media containing 30 µg/ml kanamycin sulfate and the culture was grown in a flask shaker at 37°C to a level of OD 600nm of 0.6-1.0, in approximately 3-5 hours. To the culture medium was added IPTG to a final concentration of 0.5 mM and the culture was grown for an additional two hours at 30°C. The cultures were harvested by centrifugation at 4°C at 10,000xG and the cell pellet collected. Non-lipidated PspA was recovered from the cell pellet.

[0112] The cell pellet was resuspended in PBS at 30g wet cell paste per liter PBS. The cell suspension was frozen and stored at -20°C. The cells were thawed to room temperature to effect lysis. DNaseI was added to the thawed material at a final concentration of 1 µg/ml and the mixture incubated for 30 minutes at room temperature, which resulted in a

decrease in viscosity of the material. The mixture was centrifuged at 4°C at 10,000xG, and the cell supernatant saved, which contained non-lipidated PspA. The pellet was washed with PBS, centrifuged at 4°C at 10,000xG and the cell supernatant pooled with the previous cell supernatant.

[0113] A MonoQ column (Pharmacia) was prepared in a volume of 1 ml per 2 ml cell supernatant. The column was washed with 2 column volumes of a buffer comprising 50mM Tris, 2mM EDTA, 1M NaCl pH 7.5, and then equilibrated with 5 to 10 column volumes of a buffer comprising 50 mM Tris, 2 mM EDTA, 10 mM NaCl pH 7.5. The cell supernatant pool was applied to the Q-Sepharose column and the flow through was collected. The column was washed with 2-5 column volumes of 50mM Tris, 2mM EDTA, 10mM NaCl pH 7.5, and the flow through pooled with the previous flowthrough.

[0114] The elution of bound proteins began with the first step of a 5-10 column volume wash with 50mM Tris, 2mM EDTA, 100mM NaCl pH 7.5. The second elution step was a 5-10 column volume wash with 50mM Tris, 2mM EDTA, 200mM NaCl pH 7.5. The non-lipidated PspA was contained in this fraction. SDS-PAGE analysis of this step is shown in Figure 15. The remaining bound contaminating proteins were removed with 50mM Tris and 2mM EDTA pH 7.5 with 300mM-1M NaCl.

[0115] A schematic of the purification process described in this Example is shown in Figure 12.

(For background information only) Immunogenicity of Recombinant Lipidated PapA

[0116] Purified recombinant lipidated PspA, prepared as described in Example 8, was tested for immunogenicity in mice and compared to that from non-lipidated PspA prepared as described in Example 9. For this study, C3A/N mice were immunized subcutaneously in the back of the neck with 0.5 ml of the following formulations at the indicated PspA antigen concentrations.

Formulation	PspA Antigen Concentration
Native PspA molecule of the RX1 strain (Native RX1)	200 ng/ml
Non-Lipidated Recombinant PspA (pPA-321-NL) Alone in PBS*	200 and 1000 ng/ml
Non-Lipidated Recombinant PspA (pPA-321-NL) Adsorbed to Alum	200 and 1000 ng/ml
Lipidated Recombinant PspA (pPA-321-L) Alone in PBS	200 and 1000 ng/ml
Lipidated Recombinant PspA (pPA0321-NL) Adsorbed to Alum*	200 and 1000 ng/ml
Alum*	0 ng/ml
PBS	0 ng/ml
*Alum was Hydrogel at a concentration of 200 µg/ml	

[0117] Four mice were immunized on days 0 and 21 for each dosage of the formulations. The mice were then bled on day 35 and subsequently challenged with *S. pneumoniae* of A66 strain. The days of survival after challenge for the mice were recorded and surviving mice were bled on days 36, 37, 42 and 46. From these subsequent bleeds the blood was assayed for the number of colony forming units (CFU) of *S. pneumoniae*/ml. The sera taken on day 35 were assayed by ELISA for antibodies against PspA using ELISA. The days of death for the challenged mice are shown in the following table.

Survival in Immune and Non-Immune CBA/M Mice							
Group	Immunization			Efficacy			
	Antigen	dose in µg	Alum	Days to Death	p value time to death*	Alive: Dead	p value Survival*
#1A	pPA-321-L	1.0	-	4x>14	0.01	4:0 4:0	0.01
#1B	Pa-321-L	0.2	-	4x>14	0.01		0.01
#2A	pPA-321-L	1.0	+	4x>14	0.01	4:0	0.01
#23	pPA-321-L	0.2	+	4x>14	0.01	4:0	0.01
#3A	pPR-321-NL	1.0	-	1,1,2,2	n.s.	0:4	n.s.
#3B	pPA-321 -NL	0.2	-	1,1,2, ≥15	n.s.	1:3	n.s.
#4A	pPA-321-NL	1.0	+	4x>14	0.01	4:0	0.01
#4B	pPA-321-NL	0.2	+	4x>14	0.01	4:0	0.01

(continued)

Survival in Immune and Non-Immune CBA/M Mice							
Group	Immunization			Efficacy			
	Antigen	dose in μ g	Alum	Days to Death	p value time to death*	Alive: Dead	p value Survival*
#5	FL-R $\times$ 1	0.2	-	4x>14	0.01	4:0	0.01
#6	none	0.0	+	1,1,3,6	n.s.	0:4	n.s.
#7	none	0.0	-	1,1,1, $\geq$ 15	n.s.	1:3	n.s.
	Pooled none	0.0		5x1,3,6, $\geq$ 15	--	1:7	
Note: *indicates versus pooled controls; time to death, by one tailed two sample rank test; survival, by one tailed Fisher Exact test. Calculations have been done using "one tail" since we have never observed anti-PspA immunity to consistently cause susceptibility.							

[0118] The number of CFU in the blood of the mice are shown in the table below.

Bacteremia in Immune and Non-Immune CBA/N Mice							
Group	Immunization			Cog ₁₀ CFU			
	Antigen	dose in μ g	Alum	1 day	2 day	6 day	7 day
#1A	pPA-321-L	1.0	-	$\leq$ 1.6, 1.9, 2.1, 2.5	4x $\leq$ 1.6	4x $\leq$ 1.6	n.d.
#1B	pPA-321-L	0.2	-	3x $\leq$ 1.6, 1.7	4x $\leq$ 1.6	4x $\leq$ 1.6	n.d.
#2A	pPA-321-L	1.0	+	2x $\leq$ 7.6, 1.7, 2.9	3x $\leq$ 1.6, 1.7	4x $\leq$ 1.6	n.d.
#2B	pPA-321-L	0.2	+	2x $\leq$ 1.6, 1.7, 1.7	4x $\leq$ 1.6	4x $\leq$ 1.6	n.d.
#3A	pPA-321-NL	1.0	-	$\leq$ 1.6, 1.7, d, d	d,d,d,d	d,d,d,d	d,d,d,d
#3B	pPA-321-NL	0.2	-	2x>7,d,d	$\leq$ 1.6, d,d,d	$\leq$ 1.6,d,d,d	n.d-,d,d,d
#4A	pPA-321-NL	1-0	+	2x $\leq$ 1-6, 6.7, >7	3x $\leq$ 1.6, 1.7	4x $\leq$ 1.6	n.d.
#4B	pPA-321-NL	0.2	+	$\leq$ 1.6, 1.7, 2.1 2-4	4x $\leq$ 1.6	4x $\leq$ 1-6	n.d.
#5	FL-Rx1	0.2	-	2x $\leq$ 1.6, 2.6, 2.7	4x $\leq$ 1.6	4x $\leq$ 1.6	n.d.
#6	none	0.0	+	$\leq$ 1.6, 4.1,>7,d	$\leq$ 1.6, 5-1,d,d	6.1, d,d,d	d,d,d,d
#7	none	0.0	-	1-7, >7, >7,d	$\leq$ 1.6, d,d,d	$\leq$ 1.6, d,d,d	n-d-,d,d,d
	Pooled none	0.0		$\leq$ 1.6, 4.1, >7, >7, d	2x $\leq$ 1.6, 5.1, d,d,d,d,d	$\leq$ 1.6, 6.1 d,d,d, d,d,d	n.d., d d,d,d,d,d
Note: 1 colony at the highest concentration of blood calculated out to 47 CFU or Log 1.7. Thus " $\leq$ 1.6" indicates no colonies counted $>10^7$ indicates that the mouse was alive but the number of colonies at the highest dilution was too high to count. "d" indicates the mice had died prior to assay.							

[0119] These results indicate that the recombinant protein was not protective when injected alone. The recombinant antigen adjuvanted with alum and/or PAM₃cys lipidation was immunogenic and protective. The native full length PspA antigen did not need an adjuvant to be protective. The CFU results indicate that mice protected by immunization cleared the challenging *S. pneumoniae* from the blood in two days.

[0120] ELISA analysis of sera taken on day 35 indicated that there was a good correlation between protection of the mice from *S. pneumoniae* challenge and the induction of measurable antibody responses. No detectable antibody responses were observed in the sera of mice immunized with the non-lipidated antigen (pPA-321-NL) in saline or to the negative controls that did not contain PspA antigen, (as shown in the table below). Good antibody responses were detected to the Native RX1 PspA antigen and to the recombinant PspA when it was lipidated with PAM₃cys and/or

adsorbed to alum.

ELISA Analysis of Day 35 Mouse Sera								
PspA Antigen	Alum Adsorption	PspA Dose ($\mu\text{g}/\text{mouse}$)	Resulting OD at Indicated Dilution of the Antisera*					
			600	1200	2400	4800	9600	19200
pPA-321-L	No	0.1	0.885 (0.082)	0.497 (0.043)	0.271 (0.025)	0.146 (0.017)	0.075 (0.012)	0.039 (0.009)
pPA-321-L	No	0.5	1.857 (0.060)	1.437 (0.137)	1.108 (0.150)	0.750 (0.139)	0.459 (0.092)	0.284 (0.009)
pPA-321-L	Yes	0.1	1.373 (0.325)	1.048 (0.376)	0.745 (0.362)	0.490 (0.304)	0.288 (0.197)	0.171 (0.147)
pPA-321-L	Yes	0.5	1.202 (0.162)	0.787 (0.184)	0.472 (0.187)	0.296 (0.102)	0.162 (0.061)	0.087 (0.035)
pPA-321-NL	No	0.1	0.022 (0.035)	0.030 (0.060)	0.014 (0.024)	0.007 (0.018)	0.006 (0.005)	0.001 (0.001)
pPA-321-NL	No	0.5	0.029 (0.035)	0.014 (0.014)	0.008 (0.007)	0.003 (0.004)	0.002 (0.002)	0.002 (0.002)
pPA-321-NL	Yes	0.1	0.822 (0.181)	0.481 (0.166)	0.278 (0.085)	0.154 (0.051)	0.082 (0.029)	0.042 (0.015)
pPA-321-NL	Yes	0.5	1.017 (0.139)	0.709 (0.128)	0.447 (0.101)	0.253 (0.057)	0.141 (0.034)	0.075 (0.020)
Native RX1	No	0.1	1.367 (0.084)	1.207 (0.060)	0.922 (0.070)	0.608 (0.077)	0.375 (0.048)	0.209 (0.029)
None	No	0	0.018 (0.003)	0.012 (0.008)	0.009 (0.003)	0.005 (0.002)	0.005 (0.002)	0.005 (0.002)
None	Yes	0	0.013 (0.006)	0.009 (0.008)	0.004 (0.004)	0.004 (0.003)	0.001 (0.001)	0.000 (0.000)
*The OD is the mean of the result of the four tested animals and the standard deviation is in parentheses.								

[0121] To determine whether protection was at least in part mediated by the anti-PspA antibody responses, a passive experiment was run. BALB/c mice were immunized with 0.5 μg of recombinant lipidated PspA alone or adsorbed to alum, or with recombinant non-lipidated PspA adsorbed to alum on days 0 and 21; and were bled on day 35. The antisera were diluted 1:3 or 1:15 in saline and 0.1 ml of the dilution was injected i.p. into two mice for each dilution. A 1/3 dilution of normal BALB/c mouse serum was used as a negative control. Subsequently one hour after passive immunization, the animals were challenged i.v. with the WU2 strain of *S. pneumoniae* (15,000 CFU). Mice passively immunized with anti-PspA sera were protected as compared to those mice that received dilutions of normal mouse sera as shown in the following table.

Passive Protection of BALB/c to WU2				
Immunizing Formulation PspA Antigen Alum		PspA Dose ($\mu\text{g}/\text{animal}$)	Dilution Of Serum	Day to Death Post Challenge
pPA-321-L	No	0.5	3	4, >7
			15	2, 4
pPA-321-L	Yes	0.5	3	>7, >7
			15	4, >7
PPA-321-NL	Yes	0.5	3	2, 4
			15	>7, >7

(continued)

Passive Protection of BALB/c to WU2				
Immunizing Formulation PspA Antigen Alum		PspA Dose (μg/animal)	Dilution Of Serum	Day to Death Post Challenge
None	No	0	3	2,2

Claims

1. A hybrid nucleic acid molecule, comprising a first nucleic acid sequence encoding a signal sequence of a lipoprotein and a second nucleic acid sequence encoding a mature protein, which is heterologous to the lipoprotein encoded by said first nucleic acid sequence, said first nucleic acid sequence being contiguous with said second nucleic acid sequence when the mature protein is naturally lipidated, or said first and second nucleic acid sequence being separated by one codon coding for one amino acid when the mature protein is not naturally lipidated; wherein said signal sequence is the signal sequence of an OspA protein of a *Borrelia* species and wherein said mature protein is an OspC lipoprotein of a *Borrelia* species.
2. The hybrid nucleic acid molecule of claim 1 wherein said first nucleic acid sequence and said second nucleic acid sequence are coupled in a translational open reading frame relationship.
3. The hybrid molecule of claim 1 or claim 2 wherein said OspC lipoprotein is that of a strain of *B. burgdorferi*.
4. The hybrid molecule of claim 3 wherein said strain of *B. burgdorferi* is selected from the OspC sub-type families.
5. The hybrid molecule of claim 1 wherein said OspA protein is that of a strain of *B. burgdorferi*.
6. The hybrid molecule of claim 5 wherein said strain of *B. burgdorferi* is selected from the B31, ACA1 and Ip90 families of strains.
7. An expression vector containing the hybrid nucleic acid molecule of claim 1 under control of a promoter for expression of said mature protein.
8. A method for forming a recombinant protein, which comprises;
 - incorporating the expression vector of claim 7 into a non-animal host organism; and
 - effecting expression of said mature protein from the non-animal host organism.
9. The method of claim 8 wherein said non-animal host organism is *E. coli*.
10. A process for the production of a recombinant lipoprotein, which comprises:
 - constructing a hybrid nucleic acid molecule comprising a first nucleic acid sequence encoding a signal sequence of a lipoprotein and a second nucleic acid sequence encoding a mature protein, which is heterologous to the lipoprotein encoded by said first nucleic acid, said second nucleic acid sequence being contiguous with said first sequence; wherein said signal sequence is the signal sequence of an OspA protein of a *Borrelia* species and wherein said mature protein is an OspC lipoprotein of a *Borrelia* species,
 - forming an expression vector containing said hybrid nucleic acid molecule under control of a promoter for expression of said mature protein;
 - incorporating said expression vector into a non-animal host organism;
 - effecting expression of said recombinant lipoprotein by said non-animal host organism;
 - lysing the cells of the non-animal host organism;
 - treating the lysed cells with a surfactant which selectively solubilizes said recombinant lipoprotein in preference to bacterial and other proteins and which is able to effect phase separation of a detergent phase under mild conditions;
 - effecting phase separation at a detergent phase containing solubilized recombinant lipoprotein, an aqueous phase containing bacterial and other proteins and a solid phase containing cell residue;

separating and recovering said detergent phase from said solid phase and said aqueous phase;
 contacting said detergent phase with a first chromatographic column under conditions which result in binding
 of protein other than said recombinant lipoprotein to said column to provide a flow-through from said first chro-
 matographic column containing the recombinant lipoprotein said recovering said flow-through from said first
 chromatographic column;
 contacting the flow-through from said first chromatographic column with a second chromatographic column
 under conditions which result in binding of the recombinant lipoprotein to the second chromatographic column
 in preference to contaminant proteins and lipopolysaccharides which pass through said second chromatographic
 column;
 eluting said recombinant lipoprotein from said second chromatographic column to provide an eluant containing
 said recombinant lipoprotein substantially free from lipopolysaccharide and contaminant proteins; and
 recovering from eluant.

11. The process of claim 10 wherein said first nucleic acid sequence and said second nucleic acid sequence are coupled
 in a translational open reading frame relationship.

12. The process of claim 11 wherein said surfactant is TRITON™ X-114.

13. The process of claim 12 wherein said treating of lysed cells is affected at a temperature of about 0°C to about 10°C,
 the resulting mixture is treated to a mildly elevated temperature of about 35°C to about 40°C to effect separation of
 said detergent phase, and said detergent phase is separated from said aqueous phase and said solid phase by
 centrifugation.

14. The process of claim 12 wherein said first chromatographic column is further contacted with a buffer medium at a
 pH to provide liquid containing the recombinant OspC lipoprotein from the first chromatographic column while the
 other proteins are retained on the first chromatographic column and the flow-through from the further contact is
 collected and combined with that from the first contacting step on said first chromatographic column and the combined
 flow-through is contacted with said second chromatographic column.

15. The process of claim 10 wherein said non-animal host organism lysis is effected by freezing and thawing the non-
 animal host organism.

16. Recombinantly-produced, isolated and purified lipoprotein produced by the process of claim 10, wherein the lipo-
 protein comprises the signal sequence of an OspA protein of a *Borrelia* species and a mature OspC lipoprotein of
 a *Borrelia* species.

17. A lipoprotein fusion vector PLF100 having ATCC Accession No. 69750.

18. The use of a composition comprising the lipoprotein of claim 16 in the preparation of a medicament for the prophylaxis
 or treatment of Lyme disease.

19. A composition for inducing an immunological response comprising the lipoprotein of claim 16.

Patentansprüche

1. Hybrides Nukleinsäuremolekül, welches eine erste Nukleinsäuresequenz, die eine Signalsequenz eines Lipoproteins
 codiert, und eine zweite Nukleinsäuresequenz, die ein reifes Protein codiert, welches zu dem von der ersten Nu-
 kleinsäuresequenz codierten Lipoprotein heterolog ist, umfaßt, wobei die erste Nukleinsäuresequenz an die zweite
 Nukleinsäuresequenz angrenzt, wenn das reife Protein natürlich lipidiert ist, oder die erste und die zweite Nuklein-
 säuresequenz durch ein eine Aminosäure codierendes Codon getrennt sind, wenn das reife Protein nicht natürlich
 lipidiert ist, wobei die Signalsequenz die Signalsequenz eines OspA-Proteins einer *Borrelia*-Spezies ist und wobei
 das reife Protein ein OspC-Lipoprotein einer *Borrelia*-Spezies ist.

2. Hybrides Nukleinsäuremolekül nach Anspruch 1, wobei die erste Nukleinsäuresequenz und die zweite Nukleinsäu-
 resequenz in einem Verhältnis eines für eine Translation offenen Leserasters miteinander verbunden sind.

3. Hybrides Molekül nach Anspruch 1 oder Anspruch 2, wobei das OspC-Lipoprotein dasjenige eines Stammes von

B. burgdorferi ist.

4. Hybrides Molekül nach Anspruch 3, wobei der Stamm von *B. burgdorferi* aus den OspC-Subtyp-Familien ausgewählt ist.

5. Hybrides Molekül nach Anspruch 1, wobei das OspA-Protein dasjenige eines Stammes von *B. burgdorferi* ist.

6. Hybrides Molekül nach Anspruch 5, wobei der Stamm von *B. burgdorferi* aus den Stamm-Familien B31, ACA1 und Ip90 ausgewählt ist.

7. Expressionsvektor, welcher das hybride Nukleinsäuremolekül nach Anspruch 1 unter der Kontrolle eines Promotors für die Expression des reifen Proteins enthält.

8. Verfahren zur Herstellung eines rekombinanten Proteins, welches folgendes umfaßt:

Einbringen des Expressionsvektors nach Anspruch 7 in einen nicht-tierischen Wirtsorganismus und Bewirken der Expression des reifen Proteins von dem nicht-tierischen Wirtsorganismus.

9. Verfahren nach Anspruch 8, wobei der nicht-tierische Wirtsorganismus *E. coli* ist.

10. Verfahren zur Herstellung eines rekombinanten Lipoproteins, welches folgendes umfaßt:

Konstruieren eines hybriden Nukleinsäuremoleküls, welches eine erste Nukleinsäuresequenz, die eine Signalsequenz eines Lipoproteins codiert, und eine zweite Nukleinsäuresequenz, die ein reifes Protein codiert, welches zu dem von der ersten Nukleinsäuresequenz codierten Lipoprotein heterolog ist, umfaßt, wobei die zweite Nukleinsäuresequenz an die erste Sequenz angrenzt, wobei die Signalsequenz die Signalsequenz eines OspA-Proteins einer *Borrelia*-Spezies ist und wobei das reife Protein ein OspC-Lipoprotein einer *Borrelia*-Spezies ist, Bilden eines Expressionsvektors, welcher das hybride Nukleinsäuremolekül unter der Kontrolle eines Promotors für die Expression des reifen Proteins enthält,

Einbringen des Expressionsvektors in einen nicht-tierischen Wirtsorganismus, Bewirken der Expression des rekombinanten Lipoproteins durch den nicht-tierischen Wirtsorganismus, Lysieren der Zellen des nicht-tierischen Wirtsorganismus, Behandeln der lysierten Zellen mit einem oberflächenaktiven Stoff, welcher das rekombinante Lipoprotein bevorzugt gegenüber bakteriellen und anderen Proteinen selektiv löst und welcher in der Lage ist, eine Phasentrennung einer Detergensphase unter milden Bedingungen zu bewirken, Bewirken einer Phasentrennung mit einer Detergensphase, welche gelöstes rekombinantes Lipoprotein enthält, einer wäßrigen Phase, die bakterielle und andere Proteine enthält, und einer festen Phase, die Zellrückstände enthält,

Abtrennen und Gewinnen der Detergensphase von der festen Phase und der wäßrigen Phase, Inkontaktbringen der Detergensphase mit einer ersten Chromatographiesäule unter Bedingungen, die zur Bindung von Proteinen, die nicht das rekombinante Lipoprotein sind, an die Säule führen, um einen Durchfluß von der ersten Chromatographiesäule zu liefern, der das rekombinante Lipoprotein enthält, und Gewinnen des Durchflusses aus der ersten Chromatographiesäule,

Inkontaktbringen des Durchflusses aus der ersten Chromatographiesäule mit einer zweiten Chromatographiesäule unter Bedingungen, die zur Bindung des rekombinanten Lipoproteins an die zweite Chromatographiesäule bevorzugt gegenüber verunreinigenden Proteinen und Lipopolysacchariden, die durch die zweite Chromatographiesäule hindurchströmen, führen,

Eluieren des rekombinanten Lipoproteins aus der zweiten Chromatographiesäule unter Bereitstellung eines Eluats, welches das rekombinante Lipoprotein enthält, das im wesentlichen frei von Lipopolysaccharid und verunreinigenden Proteinen ist, und Gewinnen aus dem Eluat.

11. Verfahren nach Anspruch 10, wobei die erste Nukleinsäuresequenz und die zweite Nukleinsäuresequenz in einem Verhältnis eines für eine Translation offenen Leserasters miteinander verbunden sind.

12. Verfahren nach Anspruch 11, wobei der oberflächenaktive Stoff TRITON™X-114 ist.

13. Verfahren nach Anspruch 12, wobei das Behandeln der lysierten Zellen bei einer Temperatur von etwa 0°C bis etwa

10°C bewirkt wird, das resultierende Gemisch bis zu einer leicht erhöhten Temperatur von etwa 35°C bis etwa 40°C behandelt wird, um die Abtrennung der Detergensphase zu bewirken, und die Detergensphase von der wäßrigen Phase und der festen Phase mittels Zentrifugation abgetrennt wird.

- 5 14. Verfahren nach Anspruch 12, wobei die erste Chromatographiesäule weiterhin mit einem Puffermedium bei einem pH-Wert, der eine Flüssigkeit liefert, die das rekombinante OspC-Lipoprotein aus der ersten Chromatographiesäule enthält, in Kontakt gebracht wird, während die anderen Proteine auf der ersten Chromatographiesäule gehalten werden und der Durchfluß aus dem weiteren Inkontaktbringen gesammelt und mit demjenigen von der ersten Stufe des Inkontaktbringens auf der ersten Chromatographiesäule vereinigt wird, und der vereinigte Durchfluß mit der
10 zweiten Chromatographiesäule in Kontakt gebracht wird.
15. Verfahren nach Anspruch 10, wobei die Lyse des nicht-tierischen Wirtsorganismus durch Einfrieren und Auftauen des nicht-tierischen Wirtsorganismus bewirkt wird.
- 15 16. Rekombinant erzeugtes, isoliertes und gereinigtes Lipoprotein, hergestellt nach dem Verfahren von Anspruch 10, wobei das Lipoprotein die Signalsequenz eines OspA-Proteins einer *Borrelia*-Spezies und ein reifes OspC-Lipoprotein einer *Borrelia*-Spezies umfaßt.
- 20 17. Lipoprotein-Fusionsvektor PLF100 mit der ATCC-Hinterlegungsnummer 69750.
18. Verwendung einer Zusammensetzung, welche das Lipoprotein nach Anspruch 16 umfaßt, bei der Herstellung eines Medikaments für die Prophylaxe gegen oder die Behandlung von Borreliose.
- 25 19. Zusammensetzung zum Induzieren einer Immunantwort, welche das Lipoprotein nach Anspruch 16 umfaßt.

Revendications

- 30 1. Molécule hybride d'acide nucléique, comprenant une première séquence d'acide nucléique codant pour une séquence signal d'une lipoprotéine et une seconde séquence d'acide nucléique codant pour une protéine mature, qui est hétérologue de la lipoprotéine codée par ladite première séquence d'acide nucléique, ladite première séquence d'acide nucléique étant contiguë à ladite seconde séquence d'acide nucléique lorsque la protéine mature est naturellement lipidée, ou lesdites première et seconde séquences d'acide nucléique étant séparées par un codon codant pour un acide aminé lorsque la protéine mature n'est pas naturellement lipidée ; dans laquelle ladite séquence signal
35 est la séquence signal d'une protéine OspA d'une espèce *Borrelia* et dans laquelle ladite protéine mature est une lipoprotéine OspC d'une espèce *Borrelia*.
2. Molécule hybride d'acide nucléique selon la revendication 1, dans laquelle ladite première séquence d'acide nucléique et ladite seconde séquence d'acide nucléique sont couplées dans une relation traductionnelle dans un cadre ouvert de lecture.
- 40 3. Molécule hybride selon la revendication 1 ou la revendication 2, dans laquelle ladite lipoprotéine OspC est celle d'une souche de *B. burgdorferi*.
- 45 4. Molécule hybride selon la revendication 3, dans laquelle ladite souche de *B. burgdorferi* est choisie parmi les familles du sous-type OspC.
5. Molécule hybride selon la revendication 1, dans laquelle ladite protéine OspA est celle d'une souche de *B. burgdorferi*.
- 50 6. Molécule hybride selon la revendication 5, dans laquelle ladite souche de *B. burgdorferi* est choisie parmi les familles B31, ACA1 et Ip90 des souches.
7. Vecteur d'expression contenant la molécule hybride d'acide nucléique selon la revendication 1, sous le contrôle d'un promoteur pour l'expression de ladite protéine mature.
- 55 8. Procédé de formation d'une protéine recombinante, qui comprend :

l'incorporation du vecteur d'expression selon la revendication 7 dans un organisme hôte non animal ; et

la mise en oeuvre de l'expression de ladite protéine mature à partir de l'organisme hôte non animal.

9. Procédé selon la revendication 8, dans lequel ledit organisme hôte non animal est *E. coli*.

10. Procédé pour la production d'une lipoprotéine recombinante, qui comprend :

la construction d'une molécule hybride d'acide nucléique comprenant une première séquence d'acide nucléique codant pour une séquence signal d'une lipoprotéine et une seconde séquence d'acide nucléique codant pour une protéine mature, qui est hétérologue de la lipoprotéine codée par ledit premier acide nucléique, ladite seconde séquence d'acide nucléique étant contiguë à ladite première séquence ; dans laquelle ladite séquence signal est la séquence signal d'une protéine OspA d'une espèce *Borrelia* et dans laquelle ladite protéine mature est une lipoprotéine OspC d'une espèce *Borrelia*.

la formation d'un vecteur d'expression contenant ladite molécule hybride d'acide nucléique sous le contrôle d'un promoteur pour l'expression de ladite protéine mature ;

l'incorporation dudit vecteur d'expression dans un organisme hôte non animal ;

la mise en oeuvre de l'expression de ladite lipoprotéine recombinante par ledit organisme hôte non animal ;

la lyse des cellules de l'organisme hôte non animal ;

le traitement des cellules lysées avec un tensioactif qui solubilise sélectivement ladite lipoprotéine recombinante de préférence à des protéines bactériennes ou d'autres protéines et qui est capable de réaliser une séparation de phases d'une phase détergent dans des conditions douces ;

la réalisation de la séparation de phases en une phase détergent contenant la lipoprotéine recombinante solubilisée, une phase aqueuse contenant des protéines bactériennes et d'autres protéines, et une phase solide contenant le résidu cellulaire ;

la séparation et la récupération de ladite phase détergent d'avec ladite phase solide et ladite phase aqueuse ;

la mise en contact de ladite phase détergent avec une première colonne de chromatographie dans des conditions qui entraînent la liaison de protéines autres que ladite lipoprotéine recombinante à ladite colonne pour fournir un écoulement continu à partir de ladite première colonne de chromatographie contenant la lipoprotéine recombinante ladite récupération dudit écoulement continu provenant de ladite première colonne de chromatographie ;

la mise en contact de l'écoulement continu provenant de ladite première colonne de chromatographie avec une seconde colonne de chromatographie dans des conditions qui entraînent la liaison à la seconde colonne de chromatographie de la lipoprotéine recombinante de préférence aux protéines contaminantes et aux lipopolysaccharides qui passent à travers ladite seconde colonne de chromatographie ;

l'élution de ladite lipoprotéine recombinante de ladite seconde colonne de chromatographie pour fournir un éluant contenant ladite lipoprotéine recombinante substantiellement dépourvue de lipopolysaccharide et de protéines recombinantes ; et

la récupération à partir de l'éluant.

11. Procédé selon la revendication 10, dans lequel ladite première séquence d'acide nucléique et ladite seconde séquence d'acide nucléique sont couplées dans une relation traductionnelle dans un cadre ouvert de lecture.

12. Procédé selon la revendication 11, dans lequel ledit tensioactif est du TRITON™ X-114.

13. Procédé selon la revendication 12, dans lequel ledit traitement des cellules lysées est effectué à une température comprise entre environ 0°C et environ 10°C, le mélange résultant est traité jusqu'à une température modérément élevée comprise entre environ 35°C et environ 40°C pour effectuer la séparation de ladite phase détergent, et ladite phase détergent est séparée de ladite phase aqueuse et de ladite phase solide par centrifugation.

14. Procédé selon la revendication 12, dans lequel ladite première colonne de chromatographie est, en outre, mise en contact avec un milieu tampon à un certain pH pour fournir un liquide contenant la lipoprotéine recombinante OspC provenant de la première colonne de chromatographie tandis que les autres protéines sont retenues sur la première colonne de chromatographie et l'écoulement continu provenant de la mise en contact suivante est recueilli et combiné avec celui provenant de la première étape de mise en contact sur ladite première colonne de chromatographie et l'écoulement continu combiné est mis en contact avec ladite seconde colonne de chromatographie.

15. Procédé selon la revendication 10, dans lequel ladite lyse de l'organisme hôte non animal est effectuée par congélation et décongélation de l'organisme hôte non animal.

16. Lipoprotéine produite par recombinaison, isolée et purifiée, produite par le procédé selon la revendication 10, ladite lipoprotéine comprenant la séquence signal d'une protéine OspA d'une espèce *Borellia* et une lipoprotéine mature OspC d'une espèce *Borellia*.

5 17. Vecteur PLF100 de fusion d'une lipoprotéine, ayant le numéro d'accès No. 69750.

18. Utilisation d'une composition comprenant la lipoprotéine selon la revendication 16 dans la préparation d'un médicament destiné à la prophylaxie ou au traitement de la maladie de Lyme.

10 19. Composition destinée à induire une réponse immunitaire comprenant la lipoprotéine selon la revendication 16.

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45

50

55

FIG. 1

Construction of Lipoprotein Fusion Vector pLF100

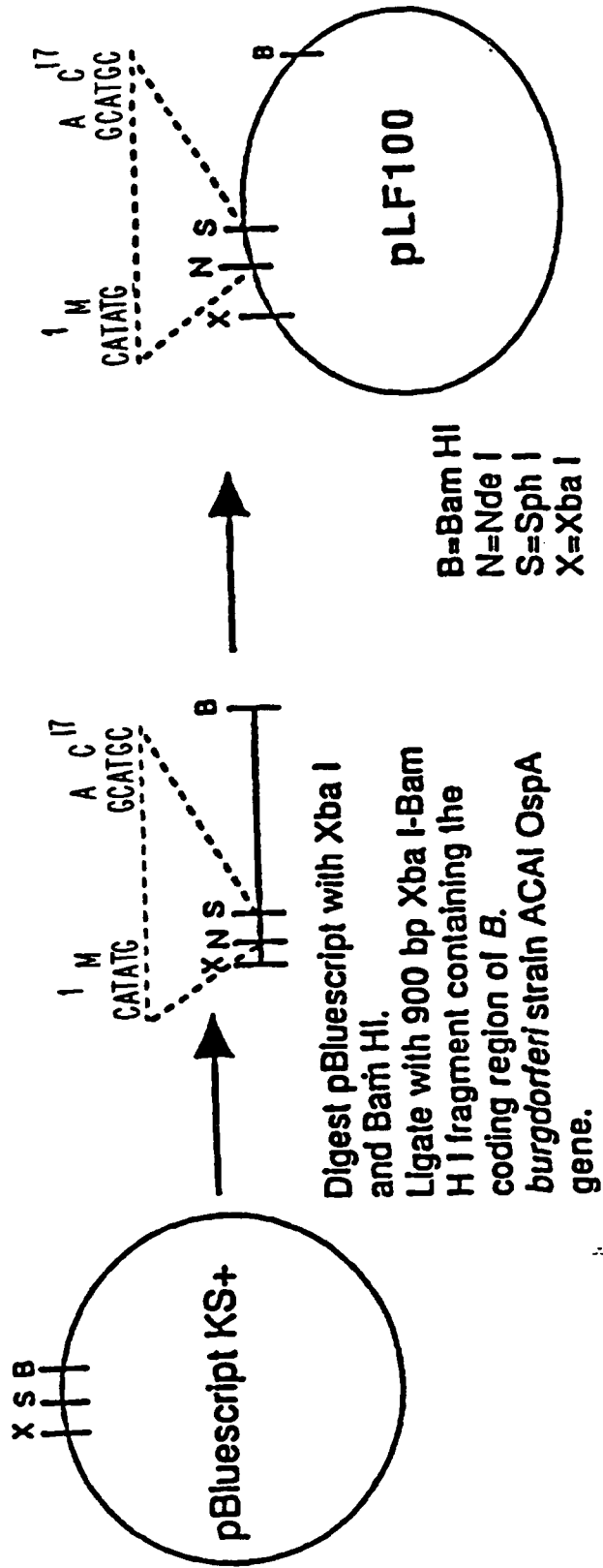


FIG. 2

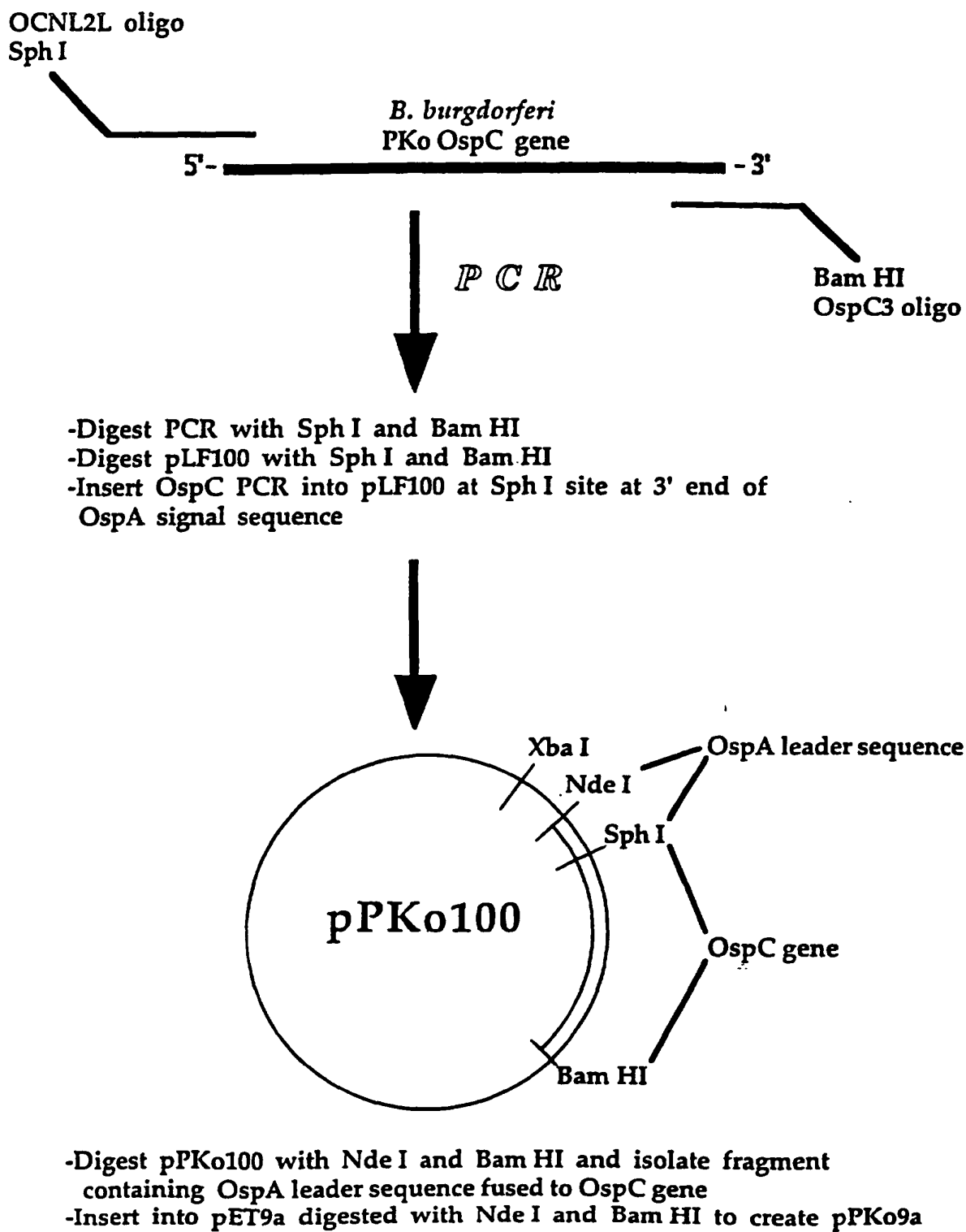


FIG. 3

- Suspend cells in PBS
- Freeze/thaw to effect cell lysis
- OspC-L extracted with 0.3% Triton X-114

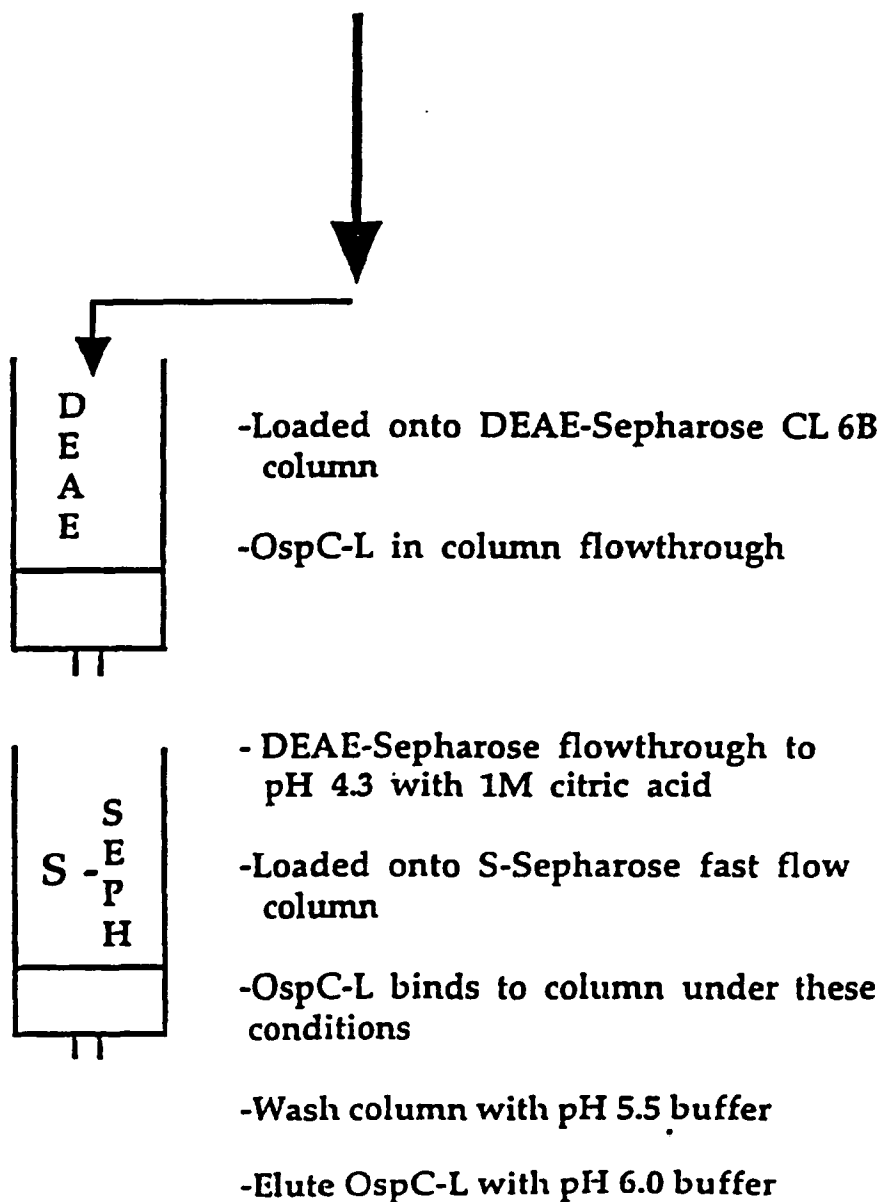


FIG. 4

-Suspend cells in lysis buffer



-Add Triton X-100 to 1% to effect cell lysis

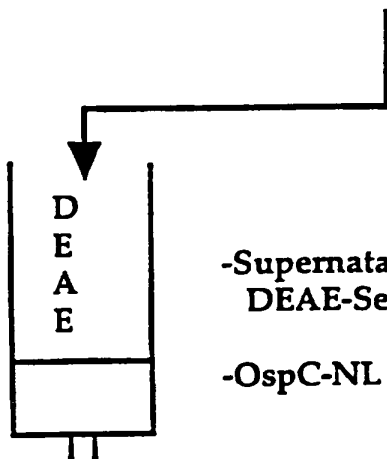


-Add NaCl to 1M final concentration



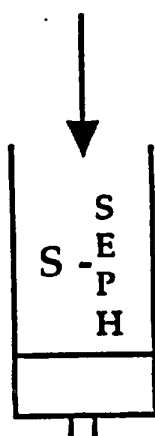
-Centrifuge at 20,000 x g, 30 min.

-Dialyze supernatant vs. 50mM TRIS, pH 8
2mM EDTA



-Supernatant loaded onto
DEAE-Sepharose CL 6B column

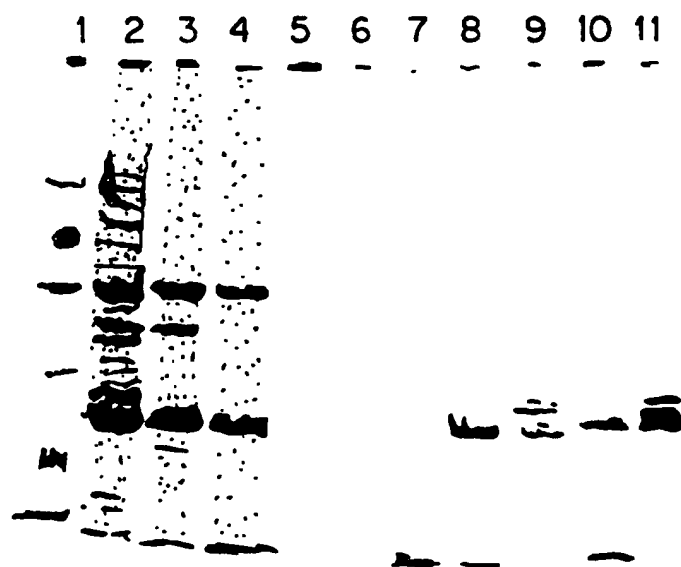
-OspC-NL in column flowthrough



-Dialyze DEAE-Sepharose flowthrough vs.
0.1 M phosphate buffer, pH 6.0

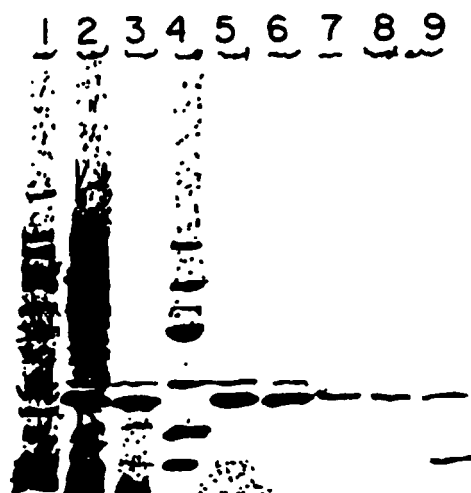
- DEAE-Sepharose flowthrough dialysate
loaded onto S-Sepharose fast flow column

-Purified OspC-NL eluted in dialysis buffer +
0.15 M NaCl

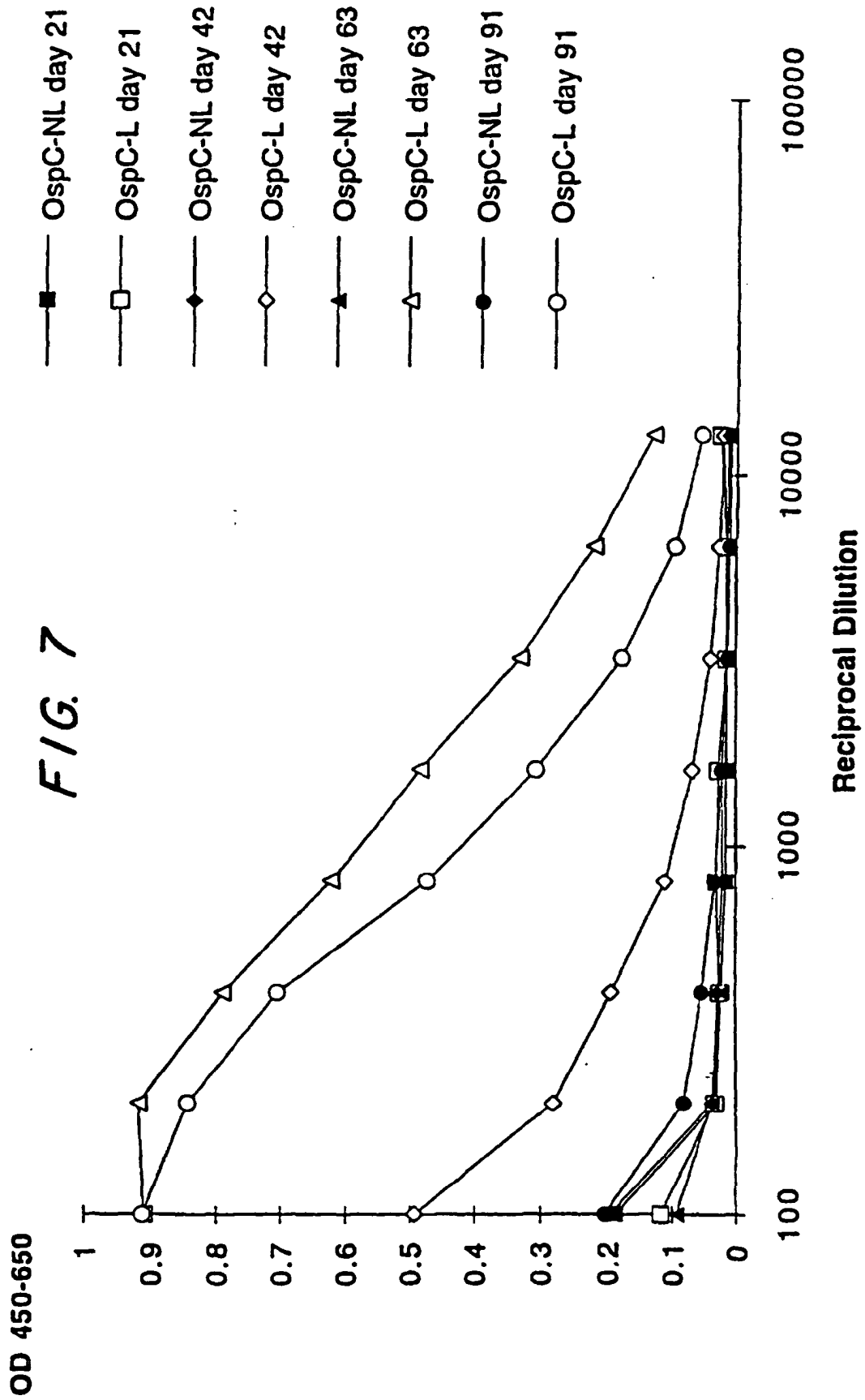
FIG. 5

- 1 - Low molecular weight standards
- 2 - OspC-L/BL21 lysate
- 3 - OspC-L/BL21 detergent phase
- 4 - OspC-L/BL21 DEAE flow through/wash pool
- 5 - OspC-L/BL21 S-Sepharose flow through
- 6 - OspC-L/BL21 S-Sepharose wash
- 7 - OspC-L/BL21 S-Sepharose elutant pH 5.5
- 8 - OspC-L/BL21 S-Sepharose elutant pH 6.0
- 9 - OspC-L/BL21 S-Sepharose elutant pH 7.5
- 10 - OspC-L/BL21 S-Sepharose elutant pH 5.7
- 11 - OspC-L/BL21 S-Sepharose elutant pH 7.5

FIG. 6



- 1 - OspC-NL/JM109 T= 0
- 2 - OspC-NL/JM109 T= 3 hours
- 3 - OspC-NL/JM109 DEAE flow through/wash pool
- 4 - Low molecular weight standards
- 5 - OspC-NL/JM109 S-Sepharose load
- 6 - OspC-NL/JM109 S-Sepharose flow through
- 7 - OspC-NL/JM109 S-Sepharose wash
- 8 - OspC-NL/JM109 S-Sepharose elutant 150 mM NaCl
- 9 - OspC-NL/JM109 S-Sepharose elutant 500 mM NaCl



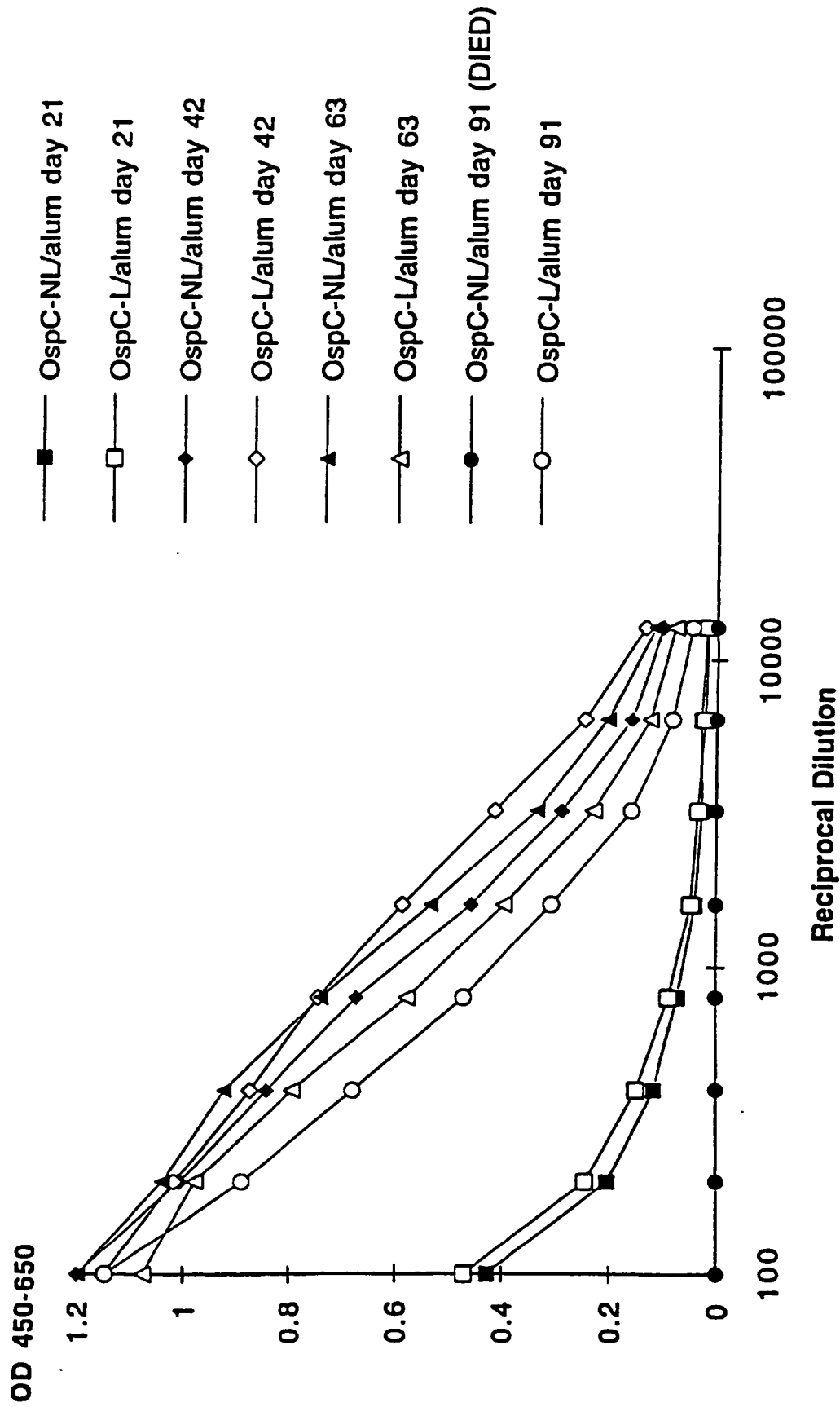
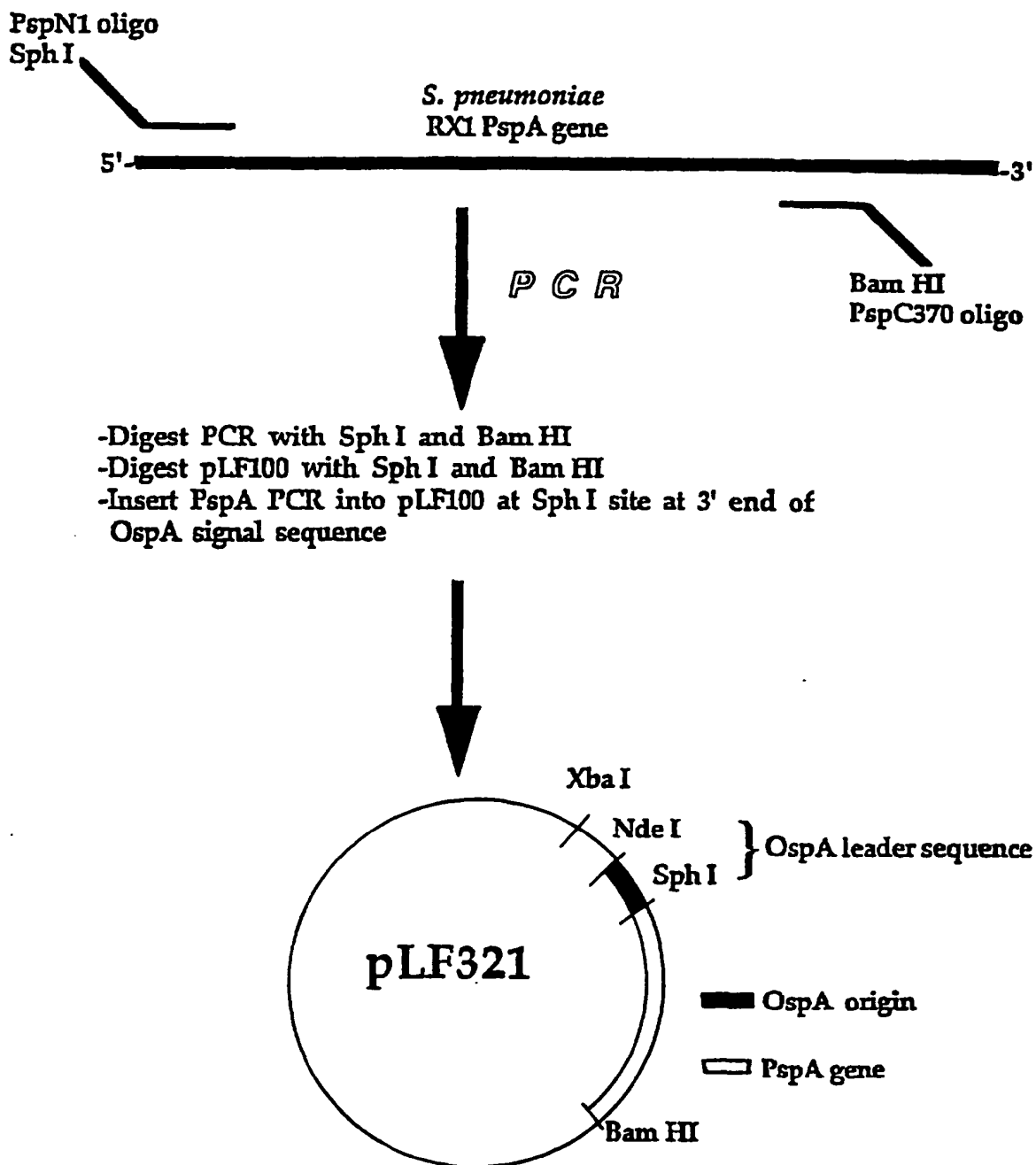


FIG. 8

FIG. 9



- Digest pLF321 with **Nde I** and **Bam HI** and isolate fragment containing OspA leader sequence fused to PspA gene
- Insert into pET9a digested with **Nde I** and **Bam HI** to create pPA321-L

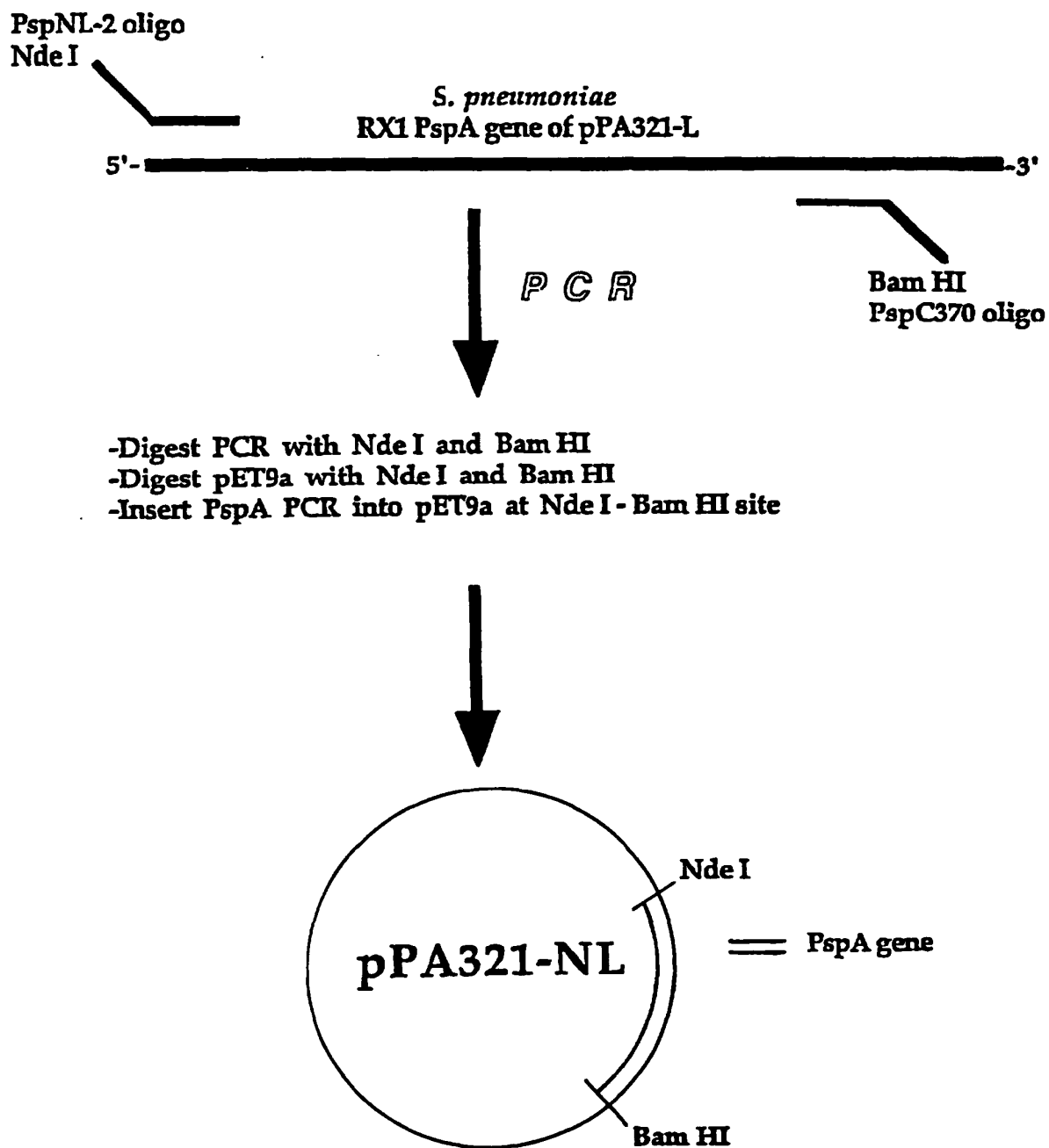


FIG. 10

PURIFICATION OF PA321-L

- Suspend cells in PBS
- Freeze/thaw to effect cell lysis
- PA321-L extracted with 0.3%-1% Triton-X114
- pH is adjusted to 4.0

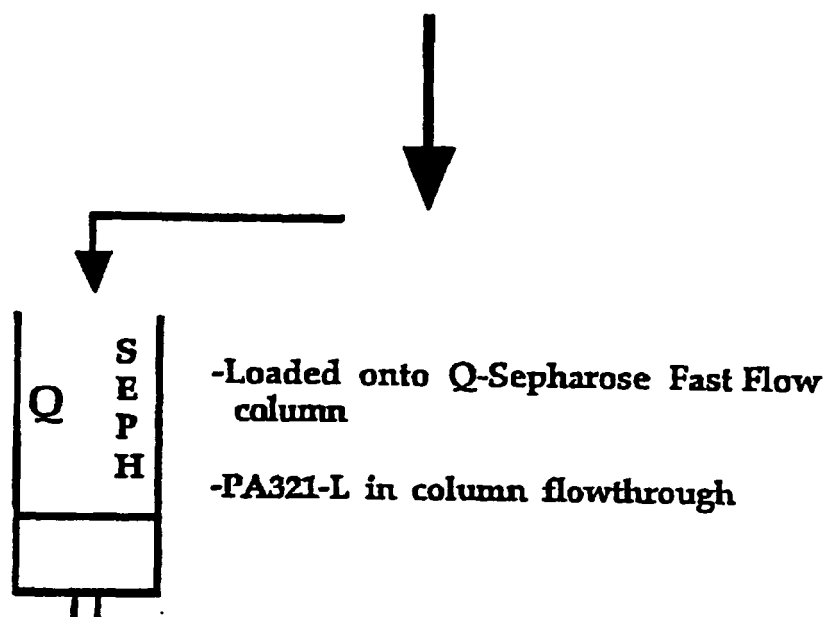
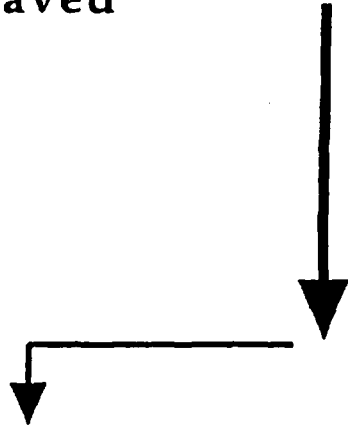
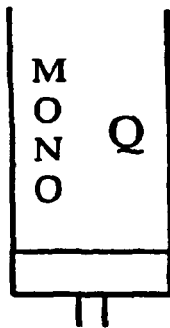


FIG. 11

- Suspend cells in PBS
- Freeze/thaw to effect cell lysis
- Cell lysate is centrifuged; cell supernant is saved



-Loaded onto a MonoQ column at pH 7.5



-Column is washed with loading buffer
(50mM Tris 2mM EDTA 10mM NaCl pH 7.5)

-Column is washed with loading buffer containing
100mM NaCl

-PA321-NL is eluted with loading buffer containing
200mM NaCl

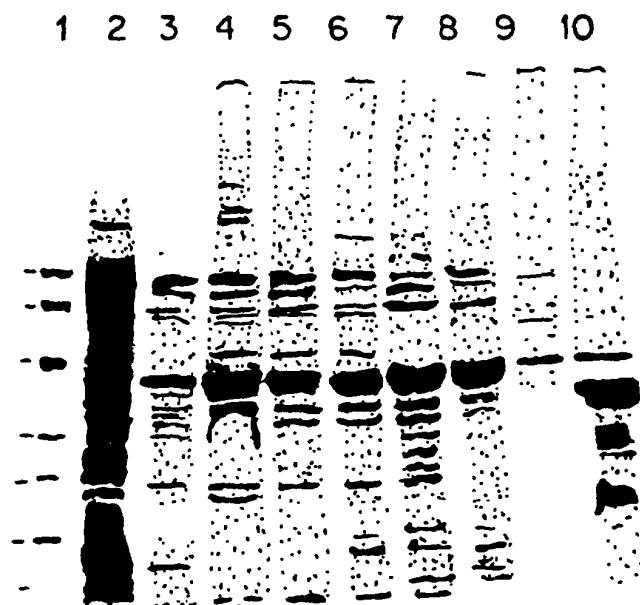
-Remaining contaminants are removed with loading
buffer containing 1M NaCl

PURIFICATION OF PA321-NL

FIG. 12

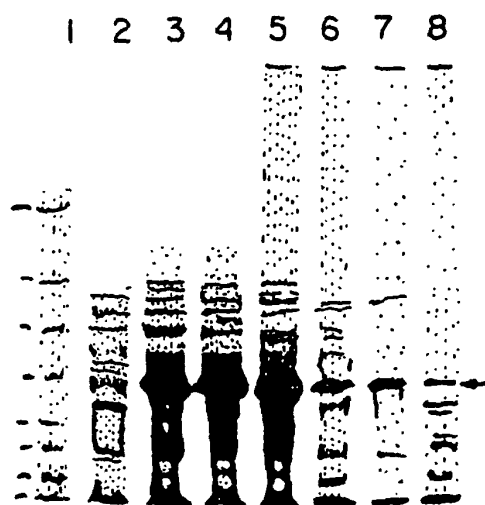
FIG. 13

PA321-L Purification Fractions



1. Prestained low molecular weight standards (106, 80, 49.5, 32.5, 27.5, and 18.5 kDa)
2. PA321-L/BL21(DE3)pLysS; T = 0
3. PA321-L/BL21(DE3)pLysS; T = 1 hour after IPTG addition
4. PA321-L/BL21(DE3)pLysS; T = 2 hours after IPTG addition
5. PA321-L/BL21(DE3)pLysS; T = 3 hours after IPTG addition
6. PA321-L/BL21(DE3)pLysS; T = 4 hours after IPTG addition
7. PA321-L/BL21(DE3)pLysS; cell lysate
8. PA321-L/BL21(DE3)pLysS; Aqueous phase
9. PA321-L/BL21(DE3)pLysS; Detergent phase
10. PA321-L/BL21(DE3)pLysS; pellet

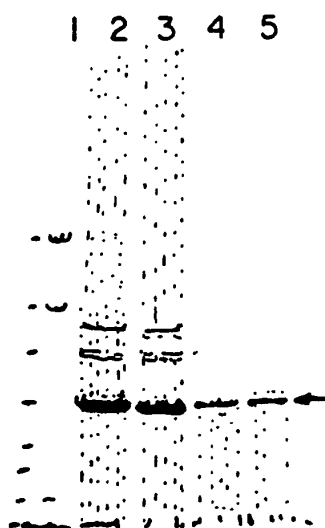
FIG. 14



PA321-NL Purification Fractions.

1. Prestained broad molecular weight standards (200, 118, 78, 47.1, 31.4, 25.5, 18.8 and 8.3 kDa)
2. PA321-NL/BL21(DE3)pLysS; T = 0
3. PA321-NL/BL21(DE3)pLysS; T = 1 hour after IPTG addition
4. PA321-NL/BL21(DE3)pLysS; T = 2 hours after IPTG addition
5. PA321-NL/BL21(DE3)pLysS; T = 3 hours after IPTG addition
6. PA321-NL/BL21(DE3)pLysS; cell lysate
7. PA321-NL/BL21(DE3)pLysS; cell supernatant and pellet wash pool
8. PA321-NL/BL21(DE3)pLysS; pellet

FIG. 15



PA321-NL and PA321-L Purification Samples

1. Prestained broad molecular weight standards (200, 118, 78, 47.1, 31.4, 25.5, 18.8, and 8.3 kDa)
2. PA321-NL MonoQ 0.2M NaCl eluent, lot# 354A-146
3. PA321-NL MonoQ 0.2M NaCl eluent, lot# 354A-147
4. PA321-L Q-sepharose pH 4.0 flow through, lot# 354A-148
5. PA321-L Q-sepharose pH 4.0 flow through, lot# 354A-150